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(71) Applicants *(for all designated States except US)*: **BROWN UNIVERSITY RESEACH FOUNDATION** [US/US]; 42 Charlesfield Street, Providence, RI 02912 (US). **HOWARD HUGHES MEDICAL INSTITUTE** [US/US]; 4000 Jones Bridge Road, Chevy Chase, MD 20815 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **ALBERINI, Cristina, M.** [US/US]; 550 Wayland Way, Providence, RI 02912 (US). **BEAR, Mark, F.** [US/US]; 244 Metacom Avenue, Bristol, RI 02806 (US).

(74) Agents: **VINCENT, Matthew, P.** et al.; Ropes & Gray, One International Place, Boston, MA 02110-2624 (US).

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(54) Title: METHODS AND COMPOSITIONS FOR REGULATING MEMORY CONSOLIDATION

(57) Abstract: The present invention is based on discovery of genes that are up- or down-regulated in inhibitory avoidance, e.g., long-term memory, which genes are therefor believed to have roles in memory consolidation. In particular, we have discovered that memory consolidation involves the regulation of expression of such genes as zif268 (EGR1), insulin-like growth factor (IGF-1), glutamate receptor (1 (GluR1), glutamate receptor 2 (GluR2), c/EBPβ and VGF. For ease of reading, these genes are collectively referred to herein as "LTM genes", and their encoded proteins as "LTM proteins".

METHODS AND COMPOSITIONS FOR REGULATING MEMORY CONSOLIDATION

Background of the Invention

Behavioral research has found that the human mind consolidates memory at certain key time intervals. The initial phase of memory consolidation occurs in the first few minutes after we are exposed to a new idea or learning experience. If a learning experience has on-going meaning to us, the next week or so serves as a further period of memory consolidation. In effect, in this phase, the material moves from short-term memory to long-term memory for storage.

It has been known for several decades that the formation of long-term memory requires gene expression. The prevailing hypothesis for the formation of long-term memory (*LTM*) is that introduction of a memory item alters the pattern of existing neuronal connectivity to form a neuronal network that will subserve the information for long-term storage. Modulation of synaptic efficacy is induced by changes in synaptic transmission within selected synapses or alteration in synaptic contacts. These changes are in turn supported by molecules that underlie transmission or synaptic remodeling. It is suggested that modulation of gene expression is needed for LTM formation to overcome the relative short lifetime of proteins in neurons (as compared with enduring memory).

In animal models of learning and memory, the requirement for de novo protein synthesis around the time of training has long been a definitive property of long-term memory that separates it from other types of memory retention. Thus, the storage of long-term memory is associated with cellular program(s) of gene expression, altered protein synthesis, and the growth of new synaptic connections. For instance, recent work suggests that this property of memory formation may have a specific molecular underpinning that involves cAMP-responsive transcription and that is mediated through the cAMP responsive element binding protein (CREB) family of transcription factors.

CREB is a nuclear protein that modulates the transcription of genes with cAMP responsive elements in their promoters. Increases in the concentration of either calcium or cAMP can trigger the phosphorylation and activation of CREB. Following its phosphorylation, CREB binds to the enhancer element CRE which is located in the upstream region of cAMP-responsive genes, thus triggering transcription. Some of the

newly-synthesized proteins are additional transcription factors that ultimately give rise to the activation of late response genes, whose products are responsible for the modification of synaptic efficacy leading to LTM.

CREB subserves the formation of memories of various types of tasks that utilize different brain structures. Evidence is available suggesting that CREB regulates the transcription of genes that subserve LTM. In Aplysia, for example, CREB activation has been interfered with by microinjection of CRE containing oligonucleotides into cultured neurons. In Drosophila, CREB function has been disrupted using a reverse genetic approach. Thus, LTM has been specifically blocked by the induced expression of a CREB repressor isoform, and enhanced by the induced expression of an activator isoform. In mouse, the role of CREB has been confirmed by behavioural analyses of a knock-out line with a targeted mutation in the CREB gene. In these mutants, learning and short term memory are normal, whereas long term memory is impaired. On the whole, the data suggest that encoding of long term memories involve highly conserved molecular mechanisms.

Animals with lesions of the medial temporal lobes and related thalamic structures show a profound disruption of memory consolidation. We have previously demonstrated that fornix-dependent lesion-induced amnesia is associated with abnormal regulation of gene expression in specific subregions of the hippocampus. See, for example, Taubenfeld et al. (1999) Nat Neurosci 2:309-10. In normal animals, inhibitory avoidance training produces a rapid and persistent increase in the phosphorylation of CREB, which is a necessary step in the regulation of CRE-mediated gene expression required for memory consolidation. The change in CREB phosphorylation is largely confined to hippocampal fields CA1 and dentate gyrus, and lasts at least 6 hours after training. Animals with fornix lesions learn the inhibitory avoidance and display memory at control levels for up to 6 hours, however, by 24 hours they exhibit amnesia. The amnesic animals also fail to exhibit any increase in hippocampal CREB phosphorylation after training. Our results suggest that hippocampal inputs passing through the fornix regulate consolidation of this form of memory via regulation of CREB-mediated gene expression in hippocampal neurons.

Initial learning is likely to result from changes in the transmission of synapses conveying information about where the animal is in space. Whether or not these

changes are made permanent depends on the timely occurrence of new gene expression. Signals impinging on hippocampal neurons via the fornix contribute to memory consolidation by modulating the gene expression required for the establishment of long-term memory. Identification of the critical chemical signals and their transduction pathways can suggest treatments for amnesia associated with damage to the temporal lobe memory system.

Summary of the Invention

One aspect of the present invention provides a a method for modulating long term memory consolidation in an animal comprising treating an animal with an agent that modulates the activity of one or more of zif 268, insulin-like growth factor, glutamate receptor 1 (GluR1), glutamate receptor 2 (GluR2), c/EBPβ and VGF.

Another aspect of the present invention relates to a method for enhancing long term memory consolidation in an animal comprising treating an animal with an agent that modulates a signal transduction pathway of glutamate receptor 1 (GluR1) or glutamate receptor 2 (GluR2), which agent is a ligand for the GluR1 or GluR2 receptor.

Still another aspect of the present invention relates to a method for identifying an agent (e.g., as a drug discovery assay) which modulates memory consolidation, comprising,

(i) providing a reaction system for detecting the activity of a product encoded by a gene selected from the group consisting of of zif 268, insulin-like growth factor, glutamate receptor 1 (GluR1), glutamate receptor 2 (GluR2), c/EBPβ and VGF;

(ii) contacting said system with a test compound; and

(iii) determining if the test compound alters the activity of the gene product.

Yet another asepect of the present invention provides a method for identifying an agent (e.g., as a drug discovery assay) which modulates memory consolidation, comprising,

(i) providing a reaction system for detecting the level of expression of a gene selected from the group consisting of of zif 268, insulin-like

growth factor, glutamate receptor 1 (GluR1), glutamate receptor 2 (GluR2), c/EBPβ and VGF;

(ii) contacting said system with a test compound; and

(iii) determining if the test compound alters the level of expression of the gene.

In certain preferred embodiments, the reaction system is a cell-free system, such as a purified protein preparation or a cell-lysate. In other embodiments, the reaction system is a whole cell system.

In preferred embodiments, the assay can be used to identify agents which modulate memory consolidation from amongst a plurality of different test agents.

In certain preferred embodiments, the test compound can be small organic molecules, e.g., those having a molecular weight less than 2500 amu.

Still another aspect of the present invention provides a method of conducting a drug discovery business comprising:

(i) identifying, by one or more of the above drug discovery assay, a test compound which the level of expression of the gene or the activity of the gene product;

(ii) conducting therapeutic profiling of agents identified in step (i), or further analogs thereof, for efficacy and toxicity in animals; and

(iii) formulating a pharmaceutical preparation including one or more agents identified in step (ii) as having an acceptable therapeutic profile.

In certain preferred embodiments, the business method includes an additional step of establishing a distribution system for distributing the pharmaceutical preparation for sale, and may optionally include establishing a sales group for marketing the pharmaceutical preparation.

Yet another aspect of the present invention provides a method of conducting a target discovery business comprising:

(i) identifying, by one or more of the above drug discovery assay, a test compound which the level of expression of the gene or the activity of the gene product;

(ii) (optionally) conducting therapeutic profiling of agents identified in step (i), or further analogs thereof, for efficacy and toxicity in animals; and

(iii). licensing, to a third party, the rights for further drug development of said identified agents.

Still another aspect of the invention provides a a pharmaceutical preparation comprising, one or more compounds identified in the above assays, formulated in a pharmaceutically acceptable excipient. In certain embodiments, the preparation can also include one or more of a neuronal growth factor, a neuronal survival factor, and/or a neuronal tropic factor.

The subject pharmaceutical preparations can also include an agent that activates CREB-dependent transcription, such as a cAMP elevating agent, e.g., a cAMP agonist activates adenylate cyclase, cAMP analog, or a cAMP phosphodiesterase inhibitor.

The subject pharmaceutical preparations can be administered to an animal as part of a treatment method for enhancing memory consolidation in an animal, or otherwise enhancing the functional performance of CNS neurons.

Yet another aspect of the invention provides a method for assessing a patient for learning and/or memory functional performance including a step of detecting the expression of, or a mutation in, one or more genes selected from the group consisting of zif 268, insulin-like growth factor, glutamate receptor 1 (GluR1), glutamate receptor 2 (GluR2), c/EBPβ and VGF, or the level of activity of the gene products thereof, (optionally) in the patient's hippocampus.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are described in the literature. See, for example, *Molecular Cloning A Laboratory Manual*, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989); *DNA Cloning*, Volumes I and II (D. N. Glover ed., 1985); *Oligonucleotide Synthesis* (M. J. Gait ed., 1984); Mullis et al. U.S. Patent No: 4,683,195; *Nucleic Acid Hybridization* (B. D. Hames & S. J. Higgins eds. 1984); *Transcription And Translation* (B. D. Hames & S. J. Higgins eds. 1984); *Culture Of Animal Cells* (R. I. Freshney, Alan R. Liss, Inc., 1987); *Immobilized Cells And Enzymes* (IRL Press, 1986); B. Perbal, *A Practical Guide To Molecular Cloning* (1984); the treatise, *Methods In Enzymology* (Academic Press, Inc., N.Y.); *Gene Transfer Vectors For Mammalian Cells* (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); *Methods In Enzymology*, Vols. 154 and 155 (Wu et al. eds.), *Immunochemical Methods In Cell And Molecular Biology* (Mayer and Walker, eds.,

Academic Press, London, 1987); *Handbook Of Experimental Immunology*, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); *Manipulating the Mouse Embryo*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986).

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

Brief Description of the Drawings

Figure 1. Time-course Northern blot analysis of zif268 and c-fos following IA training. Increase in zif268, but not c-fos mRNA is evident in all animals at 9 and 20 hr after training.

Figure 2. Time course Northern blot analysis of C/EBPβ and cyclophilin (control) mRNA following IA training in hippocampi of unoperated and fornix-lesion rats.

Figure 3. Broad scale expression profiling with cDNA expression arrays. Side by side hybridizations with cDNA probes prepared from two different RNA populations allow the simultaneous comparison of the expression levels of all the cDNAs on the array. (from Clontech user manual).

Figure 4. Examples of changes in gene array hybridizations reflecting differential expression of mRNAs following IA training. Hippocampi of control rats are compared to hippocampi of rats trained and sacrificed 9 hr later. Note that on these arrays each sequence is spotted in duplicate.

Figure 5. A Northern blot test to confirm the levels of certain transcripts.

Figure 6. Panel A. Mean escape latency for rats trained on the water maze. Rats received eight trials a day, for four consecutive days. Panel B. Mean escape latency on each of the eight trials of day one (trials 1-8) and day two (trials 9-16).

Detailed Description of the Invention

I. Overview

The present invention is based on the discovery of genes that are up- or down-regulated in inhibitory avoidance, e.g., long-term memory, which genes are therefor believed to have roles in memory consolidation. In particular, we have discovered that memory consolidation involves the regulation of expression of such genes as zif268 (EGR1), insulin-like growth factor (IGF-1), glutamate receptor 1 (GluR1), glutamate

receptor 2 (GluR2), c/EBPβ and VGF. For ease of reading, these genes are collectively referred to herein as "LTM genes", and their encoded proteins as "LTM proteins".

One aspect of the present invention relates to the use of such genes and their products for carrying out assays designed to identify agents which, by modulating the function of one or more of the LTM genes or LTM proteins, can be used to modify long term memory consolidation in animals. As described in further detail below, test agents can be assessed in a cell-based or cell-free assay for ability to inhibit or potentiate the activity of an LTM protein, e.g., by modulating an enzymatic activity of the protein, modulating the half-life of the protein, modulating the interaction of the protein with other proteins, nucleic acids, carbohydrates or other biological molecules, modulating the cellular localization of the protein and the like.

Still another aspect of the invention relates to the use of compounds identified in the subject drug screening assays for altering (increasing or decreasing) the occurrence of learning and/or memory defects in an animal, and thus, altering the learning ability and/or memory capacity of the animal. As a result, the compounds of the present invention may be useful as therapeutic agents in memory impairment, e.g., due to toxicant exposure, brain injury, epilepsy, mental retardation in children and senile dementia, including Alzheimer's disease.

II. Definitions

For convenience, the meaning of certain terms and phrases employed in the specification, examples, and appended claims are provided below.

"Cells," "host cells" or "recombinant host cells" are terms used interchangeably herein. It is understood that such terms refer not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

"Complementary" sequences as used herein refer to sequences which have sufficient complementarity to be able to hybridize, forming a stable duplex.

The "CREB" family of proteins (sometimes referred to as the ATF family) is best known for the three members that can mediate cAMP-responsive transcription: CREB itself, CREM and ATF-1 (DeGroot et al. (1993) Mol Endocrinol 7:145–153).

These basic-region, leucine-zipper proteins bind to DNA sequences, called cAMP response element (CRE) sites, which are often found in the upstream regulatory regions of genes whose synthesis is cAMP responsive. Molecular analysis has shown that CRE sites, and their interaction with CREB family members, are necessary for cAMP responsiveness. After the catalytic subunit of PKA translocates to the nucleus, it can directly phosphorylate the serine residue at position 133 on CREB, thus activating the protein and directly linking the cAMP transduction pathway to the induction of new gene expression (Backsai et al. (1993) Science 260: 222–226; and Hagiwara et al. (1993) Mol Cell Biol 13:4852–4859). CREB is also phosphorylated via other kinases, such as described above and in Deisseroth et al. (1996) Neuron 16:89-101; Impey et al. (1996) Neuron 16:973-82; and Impey et al. (1998) Neuron 21:869-883.

As is well known, genes for a particular polypeptide may exist in single or multiple copies within the genome of an individual. Such duplicate genes may be identical or may have certain modifications, including nucleotide substitutions, additions or deletions, which all still code for polypeptides having substantially the same activity. The term "DNA sequence encoding" a polypeptide may thus refer to one or more genes within a particular individual. Moreover, certain differences in nucleotide sequences may exist between individual organisms, which are called alleles. Such allelic differences may or may not result in differences in amino acid sequence of the encoded polypeptide yet still encode a protein with the same biological activity.

As used herein, the term "gene" or "recombinant gene" refers to a nucleic acid molecule comprising an open reading frame encoding a polypeptide, including both exon and (optionally) intron sequences. The term "intron" refers to a DNA sequence present in a given gene which is not translated into protein and is generally found between exons.

"Homology" or "identity" or "similarity" refers to sequence similarity between two peptides or between two nucleic acid molecules. Homology can be determined by comparing a position in each sequence which may be aligned for purposes of comparison. When a position in the compared sequence is occupied by the same base or amino acid, then the molecules are homologous at that position. A degree of homology between sequences is a function of the number of matching or homologous positions shared by the sequences. The term "percent identical" refers to sequence identity

between two amino acid sequences or between two nucleotide sequences. Identity can each be determined by comparing a position in each sequence which may be aligned for purposes of comparison. When an equivalent position in the compared sequences is occupied by the same base or amino acid, then the molecules are identical at that position; when the equivalent site occupied by the same or a similar amino acid residue (e.g., similar in steric and/or electronic nature), then the molecules can be referred to as homologous (similar) at that position. Expression as a percentage of homology/similarity or identity refers to a function of the number of identical or similar amino acids at positions shared by the compared sequences. Various alignment algorithms and/or programs may be used, including FASTA, BLAST or ENTREZ. FASTA and BLAST are available as a part of the GCG sequence analysis package (University of Wisconsin, Madison, Wis.), and can be used with, e.g., default settings. ENTREZ is available through the National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, Md. In one embodiment, the percent identity of two sequences can be determined by the GCG program with a gap weight of 1, e.g., each amino acid gap is weighted as if it were a single amino acid or nucleotide mismatch between the two sequences.

The term "interact" as used herein is meant to include detectable interactions between molecules, such as can be detected using, for example, a two hybrid assay. The term interact is also meant to include "binding" interactions between molecules. Interactions may be protein-protein or protein-nucleic acid in nature.

The term "isolated" as used herein with respect to nucleic acids, such as DNA or RNA, refers to molecules separated from other DNAs or RNAs, respectively, that are present in the natural source of the macromolecule. For example, an isolated nucleic acid encoding a particular polypeptide preferably includes no more than 10 kilobases (kb) of nucleic acid sequence which naturally immediately flanks the gene in genomic DNA, more preferably no more than 5 kb of such naturally occurring flanking sequences, and most preferably less than 1.5 kb of such naturally occurring flanking sequence. The term isolated as used herein also refers to a nucleic acid or peptide that is substantially free of cellular material, viral material, or culture medium when produced by recombinant DNA techniques, or chemical precursors or other chemicals when chemically synthesized. Moreover, an "isolated nucleic acid" is meant to include nucleic acid fragments which

are not naturally occurring as fragments and would not be found in the natural state. The term "isolated" is also used herein to refer to polypeptides which are isolated from other cellular proteins and is meant to encompass both purified and recombinant polypeptides.

The term "modulation" as used herein refers to both upregulation, i.e., stimulation, and downregulation, i.e. suppression, of a response.

The "non-human animals" of the invention include mammalians such as rodents, non-human primates, sheep, dog, cow, chickens, amphibians, reptiles, etc. Preferred non-human animals are selected from the rodent family including rat and mouse, most preferably mouse. The term "chimeric animal" is used herein to refer to animals in which the recombinant gene is found, or in which the recombinant is expressed in some but not all cells of the animal. The term "tissue-specific chimeric animal" indicates that one of the recombinant genes is present and/or expressed or disrupted in some tissues but not others.

As used herein, the term "nucleic acid" refers to polynucleotides such as deoxyribonucleic acid (DNA), and, where appropriate, ribonucleic acid (RNA). The term should also be understood to include, as equivalents, analogs of either RNA or DNA made from nucleotide analogs, and, as applicable to the embodiment being described, single (sense or antisense) and double-stranded polynucleotides.

As used herein, the term "promoter" means a DNA sequence that regulates expression of a selected DNA sequence operably linked to the promoter, and which effects expression of the selected DNA sequence in cells. The term encompasses "tissue specific" promoters, i.e. promoters, which effect expression of the selected DNA sequence only in specific cells (e.g. cells of a specific tissue). The term also covers so-called "leaky" promoters, which regulate expression of a selected DNA primarily in one tissue, but cause expression in other tissues as well. The term also encompasses non-tissue specific promoters and promoters that constitutively express or that are inducible (i.e. expression levels can be controlled).

The terms "protein", "polypeptide" and "peptide" are used interchangably herein when referring to a gene product.

The term "recombinant protein" refers to a polypeptide of the present invention which is produced by recombinant DNA techniques, wherein generally, DNA encoding a polypeptide is inserted into a suitable expression vector which is in turn used to

transform a host cell to produce the heterologous protein. Moreover, the phrase "derived from", with respect to a recombinant gene, is meant to include within the meaning of "recombinant protein" those proteins having an amino acid sequence of a native protein, or an amino acid sequence similar thereto which is generated by mutations including substitutions and deletions (including truncation) of a naturally occurring form of the protein.

"Transcriptional regulatory sequence" is a generic term used throughout the specification to refer to DNA sequences, such as initiation signals, enhancers, and promoters, which induce or control transcription of protein coding sequences with which they are operably linked. In preferred embodiments, transcription of one of the recombinant genes is under the control of a promoter sequence (or other transcriptional regulatory sequence) which controls the expression of the recombinant gene in a cell-type in which expression is intended. It will also be understood that the recombinant gene can be under the control of transcriptional regulatory sequences which are the same or which are different from those sequences which control transcription of the naturally-occurring forms of proteins.

As used herein, the term "transfection" means the introduction of a nucleic acid, e.g., an expression vector, into a recipient cell by nucleic acid-mediated gene transfer. "Transformation", as used herein, refers to a piocess in which a cell's genotype is changed as a result of the cellular uptake of exogenous DNA or RNA, and, for example, the transformed cell expresses a recombinant form of a polypeptide or, in the case of anti-sense expression from the transferred gene, the expression of a naturally-occurring form of the protein is disrupted.

As used herein, the term "transgene" means a nucleic acid sequence encoding s polypeptide or an antisense transcript thereto, which is partly or entirely heterologous, i.e., foreign, to the transgenic animal or cell into which it is introduced, or, is homologous to an endogenous gene of the transgenic animal or cell into which it is introduced, but which is designed to be inserted, or is inserted, into the animal's genome in such a way as to alter the genome of the cell into which it is inserted (e.g., it is inserted at a location which differs from that of the natural gene or its insertion results in a knockout). A transgene can include one or more transcriptional regulatory sequences

and any other nucleic acid, (e.g. as intron), that may be necessary for optimal expression of a selected nucleic acid.

A "transgenic animal" refers to any animal, preferably a non-human mammal, bird or an amphibian, in which one or more of the cells of the animal contain heterologous nucleic acid introduced by way of human intervention, such as by transgenic techniques well known in the art. The nucleic acid is introduced into the cell, directly or indirectly by introduction into a precursor of the cell, by way of deliberate genetic manipulation, such as by microinjection or by infection with a recombinant virus. The term genetic manipulation does not include classical cross-breeding, or in vitro fertilization, but rather is directed to the introduction of a recombinant DNA molecule. This molecule may be integrated within a chromosome, or it may be extrachromosomally replicating DNA. In the typical transgenic animals described herein, the transgene causes cells to express a recombinant form of a proteins, e.g. either agonistic or antagonistic forms. However, transgenic animals in which the recombinant gene is silent are also contemplated, as for example, the FLP or CRE recombinase dependent constructs described below. Moreover, "transgenic animal" also includes those recombinant animals in which gene disruption is caused by human intervention, including both recombination and antisense techniques.

As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of preferred vector is an episome, i.e., a nucleic acid capable of extra-chromosomal replication. Preferred vectors are those capable of autonomous replication and/expression of nucleic acids to which they are linked. Vectors capable of directing the expression of genes to which they are operatively linked are referred to herein as "expression vectors". In general, expression vectors of utility in recombinant DNA techniques are often in the form of "plasmids" which refer generally to circular double stranded DNA loops which, in their vector form are not bound to the chromosome. In the present specification, "plasmid" and "vector" are used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forts of expression vectors which serve equivalent functions and which become known in the art subsequently hereto.

III. Exemplary Embodiments

A. Nucleic Acids encoding LTM proteins

As described below, certain embodiments of the assays of the invention use isolated nucleic acids comprising nucleotide sequences encoding a protein involved in memory consolidation, which proteins will be herein referred to as "LTM" proteins or polypeptides. The term equivalent is understood to include nucleotide sequences encoding functionally equivalent LTM polypeptides or functionally equivalent peptides having an activity of an LTM protein such as described herein. Equivalent nucleotide sequences will include sequences that differ by one or more nucleotide substitution, addition or deletion, such as allelic variants.

Preferred nucleic acids are vertebrate LTM nucleic acids. Particularly preferred vertebrate LTM nucleic acids are mammalian. Regardless of species, particularly preferred LTM nucleic acids encode polypeptides that are at least 80% similar to an amino acid sequence of a vertebrate LTM protein. In one embodiment, the nucleic acid is a cDNA encoding a polypeptide having at least one bioactivity of the subject LTM polypeptide.

Still other preferred nucleic acids of the present invention encode an LTM polypeptide which is comprised of at least 2, 5, 10, 25, 50, 100, 150 or 200 amino acid residues. For example, preferred nucleic acid molecules for use as probes/primer or antisense molecules (i.e. noncoding nucleic acid molecules) can comprise at least about 6, 12, 20, 30, 50, 100, 125, 150 or 200 base pairs in length, whereas coding nucleic acid molecules can comprise about 300, 400, 500, 600, 700, 800, 900, 950, 975, 1000 base pairs.

Another aspect of the invention provides a nucleic acid which hybridizes under stringent conditions to a nucleic acid encoding a cloned LTM gene. Appropriate stringency conditions which promote DNA hybridization, for example, 6.0 x sodium chloride/sodium citrate (SSC) at about 45°C, followed by a wash of 2.0 x SSC at 50°C, are known to those skilled in the art or can be found in Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. For example, the salt concentration in the wash step can be selected from a low stringency of about 2.0 x SSC at 50°C. to a high stringency of about 0.2 x SSC at 50°C. In addition, the temperature in the wash step can be increased from low stringency conditions at room temperature, about 22°C, to high stringency conditions at about 65°C. Both temperature and salt may

be varied, or temperature of salt concentration may be held constant while the other variable is changed. Preferred nucleic acids have a sequence at least 75% homologous and more preferably 80% and even more preferably at least 85% homologous with an nucleic acid sequence of an LTM gene. Nucleic acids at least 90%, more preferably 95%, and most preferably at least about 98-99% homologous with a nucleic sequence of an LTM gene are of course also within the scope of the invention.

Nucleic acids having a sequence that differs from the nucleotide sequences shown in one of SEQ ID NOs: 1-X due to degeneracy in the genetic code are also within the scope of the invention. Such nucleic acids encode functionally equivalent peptides (i.e., a peptide having a biological activity of a LTM polypeptide) but differ in sequence from the sequence shown in the sequence listing due to degeneracy in the genetic code. For example, a number of amino acids are designated by more than one triplet. Codons that specify the same amino acid, or synonyms (for example, CAU and CAC each encode histidine) may result in "silent" mutations which do not affect the amino acid sequence of a LTM polypeptide. However, it is expected that DNA sequence polymorphisms that do lead to changes in the amino acid sequences of the subject LTM polypeptides will exist among mammalians. One skilled in the art will appreciate that these variations in one or more nucleotides (e.g., up to about 3-5% of the nucleotides) of the nucleic acids encoding polypeptides having an activity of a LTM polypeptide may exist among individuals of a given species due to natural allelic variation.

B. LTM proteins

Certain assays of the present invention use isolated or recombinant LTM polypeptides which are isolated from, or otherwise substantially free of other cellular proteins, especially other signal transduction factors and/or transcription factors which may normally be associated with the LTM polypeptide. The term "substantially free of other cellular proteins" (also referred to herein as "contaminating proteins") or "substantially pure or purified preparations" are defined as encompassing preparations of LTM polypeptides having less than about 20% (by dry weight) contaminating protein, and preferably having less than about 5% contaminating protein. Functional forms of the subject polypeptides can be prepared, for the first time, as purified preparations by using a cloned gene as described herein. By "purified", it is meant, when referring to a peptide or DNA or RNA sequence, that the indicated molecule is present in the substantial

absence of other biological macromolecules, such as other proteins. The term "purified" as used herein preferably means at least 80% by dry weight, more preferably in the range of 95-99% by weight, and most preferably at least 99.8% by weight, of biological macromolecules of the same type present (but water, buffers, and other small molecules, especially molecules having a molecular weight of less than 5000, can be present). The term "pure" as used herein preferably has the same numerical limits as "purified" immediately above. "Isolated" and "purified" do not encompass either natural materials in their native state or natural materials that have been separated into components (e.g., in an acrylamide gel) but not obtained either as pure (e.g. lacking contaminating proteins, or chromatography reagents such as denaturing agents and polymers, e.g. acrylamide or agarose) substance, or solutions. In preferred embodiments, purified LTM preparations will lack any contaminating proteins from the same animal from which LTM is normally produced, as can be accomplished by recombinant expression of, for example, a human LTM protein in a non-human cell.

Full length proteins or fragments corresponding to one or more particular motifs and/or domains or to arbitrary sizes, for example, at least 5, 10, 25, 50, 75, 100, 125, 150 amino acids in length are within the scope of the present invention.

For example, isolated LTM polypeptides can be encoded by all or a portion of a nucleic acid sequence shown in any of SEQ ID NOs: 1-X. Isolated peptidyl portions of LTM proteins can be obtained by screening peptides recombinantly produced from the corresponding fragment of the nucleic acid encoding such peptides. In addition, fragments can be chemically synthesized using techniques known in the art such as conventional Merrifield solid phase f-Moc or t-Boc chemistry. For example, an LTM polypeptide of the present invention may be arbitrarily divided into fragments of desired length with no overlap of the fragments, or preferably divided into overlapping fragments of a desired length. The fragments can be produced (recombinantly or by chemical synthesis) and tested to identify those peptidyl fragments which can function as either agonists or antagonists of a wild-type (e.g., "authentic") LTM protein.

Recombinant polypeptides preferred by the present invention, in addition to native LTM proteins, are encoded by a nucleic acid, which is at least 85% homologous and more preferably 90% homologous and most preferably 95% homologous with an amino acid sequence of an LTM protein. In a preferred embodiment, an LTM protein of

the present invention is a mammalian LTM protein. It will be understood that certain post-translational modifications, e.g., phosphorylation and the like, can increase the apparent molecular weight of the LTM protein relative to the unmodified polypeptide chain.

Moreover, it will be generally appreciated that, under certain circumstances, it may be advantageous to provide homologs of one of the subject LTM polypeptides which function in a limited capacity as one of either an LTM agonist (mimetic) or an LTM antagonist, in order to promote or inhibit only a subset of the biological activities of the naturally-occurring form of the protein. Thus, specific biological effects can be elicited by treatment with a homolog of limited function, and with fewer side effects relative to treatment with agonists or antagonists which are directed to all of the biological activities of naturally occurring forms of LTM proteins.

Homologs of each of the subject LTM proteins can be generated by mutagenesis, such as by discrete point mutation(s), or by truncation. For instance, mutation can give rise to homologs which retain substantially the same, or merely a subset, of the biological activity of the LTM polypeptide from which it was derived. Alternatively, antagonistic forms of the protein can be generated which are able to inhibit the function of the naturally occurring form of the protein, such as by competitively binding to a downstream or upstream member of the LTM cascade which includes the LTM protein. In addition, agonistic forms of the protein may be generated which are constitutively active. Thus, the LTM protein and homologs thereof provided by the subject invention may be either positive or negative regulators of memory consolidation.

C. Cells expressing LTM proteins

As described below, the assays of the invention may include cells transfected to express a recombinant form of the subject LTM polypeptides. The host cell may be any prokaryotic or eukaryotic cell. Thus, a nucleotide sequence derived from the cloning of mammalian LTM proteins, encoding all or a selected portion of the full-length proteir, can be used to produce a recombinant form of an LTM polypeptide via microbial or eukaryotic cellular processes. Ligating the polynucleotide sequence into a gene construct, such as an expression vector, and transforming or transfecting into hosts, either eukaryotic (yeast, avian, insect or mammalian) or prokaryotic (bacterial cells), are standard procedures used in producing other well-known proteins, e.g. MAP kinase,

p53, WT1, PTP phosphatases, SRC, and the like. Similar procedures, or modifications thereof, can be employed to prepare recombinant LTM polypeptides by microbial means or tissue-culture technology in accord with the subject invention.

D. Drug Screening Assays

According to one aspect of the present invention, LTM genes and/or LTM gene products are used for carrying out assays designed to identify agents which, by modulating the function of one or more of the LTM genes, can be used to modify long term memory consolidation in animals. As described in further detail below, test agents can be assessed in a cell-based or cell-free assay for ability to inhibit or potentiate the activity of an LTM protein. As described in the examples, the LTM genes can range from cell surface receptors and secreted proteins to transcription factors. Accordingly, the invention contemplates such drug-screening formats which detect compounds that, e.g., modulate an enzymatic activity of the LTM protein, modulate the half-life of the LTM protein, modulate the interaction of the LTM protein with other proteins, nucleic acids, carbohydrates or other biological molecules, modulate the cellular localization of the LTM protein and the like. A variety of assay formats will suffice and, in light of the present inventions, will be comprehended by a skilled artisan.

Exemplary agents which can be tested in the subject drug screening assays include small organic molecules, e.g., having a molecular weight less than 2500 amu, more preferably less than less than 1000, 750 or 500 amu. Such molecules can include peptide and non-peptide moieties, nucleic acids, carbohydrates and the like. In many embodiments, it will be desirable to repeat the assay for a plurality of different test agents. For example, the subject assays can be repeated for at least 10 different test agents, and in other embodiments, for at least 100, or even at least 1000 different test agents.

(i) Cell-Free Assays

In many drug screening programs which test libraries of compounds and natural extracts, high throughput assays are desirable in order to maximize the number of compounds surveyed in a given period of time. Assays which are performed in cell-free systems, such as may be derived with purified or semi-purified proteins, are often preferred as "primary" screens in that they can be generated to permit rapid development and relatively easy detection of an alteration in a molecular target which is mediated by

a test compound. Moreover, the effects of cellular toxicity and/or bioavailability of the test compound can be generally ignored in the in vitro system, the assay instead being focused primarily on the effect of the drug on the molecular target as may be manifest in an alteration of binding affinity with upstream or downstream elements or with intrinsic enzymatic activity. Many of the LTM proteins identified by the subject method will be amenable to some form of cell-free assay formats. Soluble proteins, be they cytoplasmic or extracellular, can be recombinantly expressed and at least partially purified, or provided as lysates, for use in cell-free assays. Membrane-associated proteins can, in certain instances, be purified in detergent or liposomes, or isolated as part of a cell membrane fraction or organelle preparation.

Accordingly, in an exemplary screening assay of the present invention, a reaction mixture is generated including the LTM protein and one or more proteins (or nucleic acids) which interact with the LTM protein, such molecules being referred to herein as "LTM-interacting partners" or "LTM-IP". Examples of LTM-IP include proteins that function upstream (including both activators and repressors of LTM activity), and proteins or nucleic acids which function downstream of the LTM polypeptide, whether they are positively or negatively regulated by it. The reaction mixture also includes one or more test compounds. Detection and quantification of complexes of the LTM protein with upstream or downstream LTM-IP provide a means for determining a compound's efficacy at inhibiting or potentiating complex formation between LTM and the LTM-IPs. The efficacy of the compound can be assessed by generating dose response curves from data obtained using various concentrations of the test compound. Moreover, a control assay can also be performed to provide a baseline for comparison. In one control assay, isolated and purified LTM polypeptide is added to a composition containing the LTM-IP, and the formation of a complex is quantitated in the absence of the test compound.

Complex formation between the LTM polypeptide and a binding partner may be detected by a variety of techniques. Modulation of the formation of complexes can be quantitated using, for example: detectably labeled proteins such as radiolabeled, fluorescently labeled, or enzymatically labeled proteins; by immunoassay; or by chromatographic detection.

Typically, it will be desirable to immobilize either LTM or its interacting partner to facilitate separation of complexes from uncomplexed forms of one or both of the proteins, as well as to accommodate automation of the assay. Binding of the LTM protein to an upstream or downstream element, in the presence and absence of a candidate agent, can be accomplished in any vessel suitable for containing the reactants. Examples include microtitre plates, test tubes, and micro-centrifuge tubes. In one embodiment, a fusion protein can be provided which adds a domain that allows the protein to be bound to a matrix. For example, glutathione-S-transferase/LTM (GST/LTM) fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, Mo.) or glutathione derivatized microtitre plates, which are then combined with a cell lysate or other preparation including the LTM-IP and the test compound, and the mixture incubated under conditions conducive to complex formation (in the absence of the test compound), e.g. at physiological conditions for salt and pH, though slightly more stringent conditions may be desired. Following incubation, the beads are washed to remove any unbound LTM-IP, and the matrix immobilized and the amount of LTM-IP in the matrix determined, or in the supernatant after the complexes are subsequently dissociated. Alternatively, the complexes can be dissociated from the matrix, separated by SDS-PAGE, and the level of LTM-IP found in the bead fraction quantitated from the gel using standard electrophoretic techniques.

Other techniques for immobilizing proteins or nucleic acids on matrices are also available for use in the subject assay. For instance, either LTM or its cognate binding partner can be immobilized utilizing conjugation of biotin and streptavidin. For instance, biotinylated LTM proteins can be prepared from biotin-NHS (N-hydroxy-succinimide) using techniques well known in the art (e.g., biotinylation kit, Pierce Chemicals, Rockford, Ill.), and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical). Alternatively, antibodies reactive with the LTM protein, but which do not interfere with binding of upstream or downstream binding partners, can be derivatized to the wells of the plate, and the LTM protein trapped in the wells by antibody conjugation. As above, preparations of an LTM-IP and a test compound are incubated in the LTM-presenting wells of the plate, and the amount of complex trapped in the well can be quantitated. Exemplary methods for detecting such complexes, in addition to those described above for the GST-immobilized complexes, include

immunodetection of complexes using antibodies reactive with the LTM binding partner, or which are reactive with the LTM protein and compete with the binding partner; as well as enzyme-linked assays which rely on detecting an enzymatic activity associated with the binding partner, either intrinsic or extrinsic activity. In the instance of the latter, the enzyme can be chemically conjugated or provided as a fusion protein with an LTM-IP. To illustrate, the LTM-IP can be chemically cross-linked or genetically fused with horseradish peroxidase, and the amount of polypeptide trapped in the complex can be assessed with a chromogenic substrate of the enzyme, e.g. 3,3'-diamino-benzadine tetrahydrochloride or 4-chloro-1-napthol. Likewise, a fusion protein comprising the polypeptide and glutathione-S-transferase can be provided, and complex formation quantitated by detecting the GST activity using 1-chloro-2,4-dinitrobenzene (Habig et al (1974) J. Biol. Chem. 249:7130).

For processes which rely on immunodetection for quantitating one of the proteins trapped in the complex, antibodies against the protein, such as anti-LTM antibodies, can be used. Alternatively, the protein to be detected in the complex can be "epitope tagged" in the form of a fusion protein which includes a second polypeptide sequence for which antibodies are readily available (e.g. from commercial sources). For instance, the GST fusion proteins described above can also be used for quantification of binding using antibodies against the GST moiety. Other useful epitope tags include mycepitopes (e.g., see Ellison et al. (1991) J. Biol. Chem. 266:21150-21157) which includes a 10-residue sequence from c-myc, as well as the pFLAG system (International Biotechnologies, Inc.) or the pEZZ-protein A system (Pharmacia, N.J.).

Other cell-free ebodiments include assays which detect an intrinsic activity of an LTM protein or a complex including an LTM protein, and identify compounds that increase or inhibit that activity. For instance, the reaction micture can be generated to include the LTP protein, a substrate for an enzymatic activity of the LTM protein, and the test agent. The rate of conversion of the substrate to product is determined, and can be compared to such control samples as the LTM proteins and substrate admixed alone. Test agents which are inhibitors of the LTM activity will decrease the rate of conversion of the substrate to product, whereas test agents that increase that rate are likely to be agonists of the LTM activity.

In preferred embodiments, the substrate is readily detectable, e.g., the conversion of substrate to product a colorimetric or fluorometric change in the reaction mixture which is detectable by spectroscopic means, or creates or destroys an epitope which is detectable by immunoassay.

(ii) Cell Based Assays

In addition to cell-free assays, such as described above, the readily available LTM proteins provided by the present invention also facilitates the generation of cell-based assays for identifying small molecule agonists/antagonists and the like. The ability of a test agent to alter the activitiy of an LTM protein in the cell may include directly detecting the formation of complexes including the LTM protein, detecting an intrinic enzymatic activity of the LTM protein, directly detecting a change in cellular localization of the LTM protein, detecting a post-translational modification to the LTM protein or a change in the stability of the LTM protein, or detecting the downstream consequence of any one of such events.

Such assays can be simple binding assays. For instance, where the LTM protein is a receptor, the assay can be used to identify compounds which bind to the receptor or effect the ability of the receptor to bind its ligand. In other embodiments, cells which are phenotypically sensitive to the presence or activity of the LTM protein, e.g., if it produces a morphological change in the cell, can be caused to over- or under-express a recombinant LTM protein in the presence and absence of a test agent of interest, with the assay scoring for modulation in LTM responses by the target cell which mediated by the test agent. As with the cell-free assays, agents which produce a statistically significant change in LTM-dependent responses (either inhibition or potentiation) can be identified. For example, the level of expression of genes or gene products which are up- or down-regulated in response to the presence or activity of an LTM protein can be detected. In preferred embodiments, the regulatory regions of such genes, e.g., the 5' flanking promoter and enhancer regions, are operably linked to a detectable marker (such as luciferase) which encodes a gene product that can be readily detected.

In the event that the LTM proteins themselves, or in complexes with other proteins, are capable of binding DNA and modifying transcription of a gene, a transcriptional based assay could be used, for example, in which an LTM-responsive regulatory sequence is operably linked to a detectable marker gene.

In yet another aspect of the invention, the subject drug screening assays can utilized the LTM proteins to generate a "two hybrid" assay (see, for example, U.S. Pat. No. *5,283,317*; Zervos et al. (1993) Cell 72:223-232; Madura et al. (1993) J. Biol. Chem. 268:12046-12054; Bartel et al. (1993) Biotechniques 14:920-924; Iwabuchi et al. (1993) Oncogene 8:1693-1696; and PCT Publication No. WO 94/10300). Briefly, the two hybrid assay relies on reconstituting in vivo a functional transcriptional activator protein from two separate fusion proteins. In particular, the method makes use of chimeric genes which express hybrid proteins. To illustrate, a first chimeric gene can be generated with the coding sequence for a DNA-binding domain of a transcriptional activator fused in frame to the coding sequence for an LTM protein. The second hybrid protein encodes a transcriptional activation domain fused in frame to another polypeptide, e.g., and LTM-IP, which binds to the LTM protein. If the two fusion proteins are able to interact, e.g., form an LTM-dependent complex, they bring into close proximity the two domains of the transcriptional activator. This proximity is sufficient to cause transcription of a reporter gene which is operably linked to a transcriptional regulatory site which is bound by the DNA-binding domain of the first fusion proteins, and expression of the reporter gene can be detected and used to score for the interaction of the LTM and sample proteins.

a. Exemplary LTM proteins: GluR1 and GluR2

In certain embodiments, the subject assays are used to identify compouds which modulate the activity of a glutamate receptor, such as GluR1 (SEQ ID Nos. 1 and 2) or GluR2 (SEQ ID Nos. 3 and 4).

The subject assays can be used to identifying agents which bind to the receptors and, e.g., mimic or potentiate the activity of the natural ligand, or which inhibit binding or signal transduction by the receptor. To illustate, for binding studies, test agents can be tested for competition with binding. For example, [^{3}H]AMPA binding can be assessed as follows: cells expressing GluR1 or GluR2 are preincubated in 50 mM Tris-HCl buffer, pH 7.4, for 30 min, and then incubated at 4°C for 4h in 100 mM buffer plus 100 mM KCSN, 70 nM [^{3}H]AMPA (53 Ci/mmol, NEN, Boston, MA) and the test agent. Binding of the radiolabeled AMPA is assessed and compared to the level of binding in the absence of the test compound. Nonspecific binding is measured in the presence of 1 mM L-glutamate.

The GluR1 and GluR2 receptors appears to be coupled to postsynaptic inositol phosphate metabolism, e.g., activating the PLC/PKC/CREB/MapK pathway, as well as cAMP-mediated pathways. Accordingly, generation of second messengers, and regulation of gene expression can be used to asses the ability of a test agent to inhibit or potentiate the activity of GluR1 or GluR2.

b. Exemplary LTM proteins: Insulin-like Growth Factor 1

IGF1 (SEQ ID No.s 7 and 8) is an extracellular protein which mediates its inductive effect on cells at least in part through binding to the IGF1 receptor (IGF1-R). The receptor has an intrinsic tyrosine kinase activity, which can be targeted in the subject assay for identifying inhibitors of the kinase activity.

Signal transduction from the receptor includes interaction with a series of proteins containing Src homology-2 (SH2) domains, including SH2-containing protein tyrosine phosphatase 2 (SHP-2) and SHC. The subject assay can be used to identify agents which inhibit the interaction of IGF1-R with SH2 domains, or which inhibit the enzymatic activity of an SH2 protein which interacts with IGF1-R. For instance, the assay can combine the tyrosine-phosphorylated IGF1-R β-subunit, at least the SH2 domain of SHP-2, and a test agent, and detect the ability of the test agent to alter the formation of complexes containing IGF1-R and SHP-2.

IGF1-R also signals through acitvation of the Jak protein tyrosine kinase family, with resultant phosphorylation of the STAT transcriptional activator factors. See, for example, Frank et al (1995) PNAS 92:7779-7783; Scharfe et al. (1995) *Blood* 86:2077-2085; Bacon et al. (1995) PNAS 92:7307-7311); and Sakatsume et al (1995) *J. Biol Chem* 270:17528-17534. Events downstream of Jak phosphorylation have also been elucidated. For example, IGF1-R can cause the phosphorylation of signal transducers and activators of transcription (STAT) proteins STAT1□, STAT2β, and STAT3, as well as of two STAT-related proteins, p94 and p95. The STAT proteins translocate to the nucleus and bind to specific DNA sequences, thus suggesting a mechanism by which IGF1-R may activate specific genes

Detection means which may be scored for in the present assay, in addition to direct detection of second messangers, such as by changes in phosphorylation, includes reporter constructs or indicator genes which include transcriptional regulatory elements responsive to the STAT proteins.

c. Exemplary LTM proteins: neuroendocrine VGF

The neurotropin-inducible gene VGF (SEQ ID Nos. 11 and 12) is expressed in neuronal and endocrine tissues. It encodes a secretory protein that is proteolytically processed in neuronal cells to low molecular mass polypeptides. In addition to targeting the second messanger induction caused by contacting cells with VGF, the subject assays can also be used to identify agents which inhibit the proteolytic processing of VGF.

d. Exemplary LTM proteins: ZIF268

Zif268 (SEQ ID Nos. 5 and 6) is also known as krox-24, egr-1, TIS 8, NGFI-A or zenk. It is a zinc-finger transcription factor which binds to a so-called "EGR1 motif", e.g., a trascriptional regulatory sequence of 5'-CGCCCCCGC or 5'-GCGTGGGCG. See, for example, Rauscher et al. (1990) Science 250:1259; and Pavletich et al. (1991) Science 252:809.

In certain embodiments, the subject assay can be a transcription based assay including a reporter gene having an EGR1 motif as part of its transcriptional regulatory sequences. Test agents can be assessed for their ability to enhance or inhibit Zif268-dependent transcription.

In other embodiments, test agents can be tested for their ability to ehance or inhibit binding of Zif268 with its EGR1 motif in a competitive binding assay, e.g, a nucleic acid including the motif.

e. Exemplary LTM proteins: C/EBPβ

In another embodiment, the target for the subject drug screening assay is the transcription factor C/EBPβ (SEQ ID Nos. 9 and 10), also referred to as the CCAAT/enhancer protein. The CCAAT/Enhancer Binding Protein (C/EBP) family belongs to the basic leucine zipper class of transcription factors. The C/EBP protein binds to the CCAAT-box (consensus $GG^{T}/_{C}CAATCT$).

As above with Zif268, C/EBP-dependent transcriptional activity or competition binding assays can be used to assessed the ability of test compounds to enhance or inhibit C/EBPβ activity.

f. Exemplary Screening and Selection Assays: Second Messenger Generation

When screening for bioactivity of test compounds, intracellular second messenger generation can be measured directly. A variety of intracellular effectors have been identified as being regulated by certain of the LTM proteins described above,

including adenylyl cyclase, cyclic GMP, phosphodiesterases, phosphoinositidase C, and phospholipase A_2, as well as a variety of ions.

In one embodiment, the detection signal is provided by detecting phosphorylation of intracellular proteins, e.g., MEKKs, MEKs, or Map kinases, or by the use of reporter constructs or indicator genes which include transcriptional regulatory elements responsive to c-fos and/or c-jun.

In another embodiment, the GTPase enzymatic activity by G proteins can be measured in plasma membrane preparations by determining the breakdown of □^{32}P GTP using techniques that are known in the art (For example, see *Signal Transduction: A Practical Approach.* G. Milligan, Ed. Oxford University Press, Oxford England). When receptors that modulate cAMP are tested, it will be possible to use standard techniques for cAMP detection, such as competitive assays which quantitate [^{3}H]cAMP in the presence of unlabelled cAMP.

Certain of the subject LTM proteins stimulate the activity of phospholipase C which stimulates the breakdown of phosphatidylinositol 4,5, bisphosphate to 1,4,5-IP3 (which mobilizes intracellular Ca++) and diacylglycerol (DAG) (which activates protein kinase C). Inositol lipids can be extracted and analyzed using standard lipid extraction techniques. DAG can also be measured using thin-layer chromatography. Water soluble derivatives of all three inositol lipids (IP1, IP2, IP3) can also be quantitated using radiolabelling techniques or HPLC.

The other product of PIP2 breakdown, DAG can also be produced from phosphatidyl choline. The breakdown of this phospholipid in response to receptor-mediated signaling can also be measured using a variety of radiolabelling techniques.

The activation of phospholipase A2 can easily be quantitated using known techniques, including, for example, the generation of arachadonate in the cell.

In the case of certain receptors, such as IGF1-R, it may be desirable to screen for changes in cellular phosphorylation. Such assay formats may be useful when the receptor of interest is a receptor kinase or phosphatase. For example, immunoblotting (Lyons and Nelson (1984) *Proc. Natl. Acad. Sci. USA* 81:7426-7430) using anti-phosphotyrosine, anti-phosphoserine or abti-phosphothreonine antibodies. In addition, tests for phosphorylation could be also useful when the receptor itself may not be a

kinase, but activates protein kinases or phosphatase that function downstream in the signal transduction pathway.

One such cascade is the MAP kinase pathway that appears to mediate both mitogenic, differentiation and stress responses in different cell types. Stimulation of growth factor receptors results in Ras activation followed by the sequential activation of c-Raf, MEK, and p44 and p42 MAP kinases (ERK1 and ERK2). Activated MAP kinase then phosphorylates many key regulatory proteins, including p90RSK and Elk-1 that are phosphorylated when MAP kinase translocates to the nucleus. Homologous pathways exist in mammalian and yeast cells. For instance, an essential part of the S. cerevisiae pheromone signaling pathway is comprised of a protein kinase cascade composed of the products of the STE11, STE7, and FUS3/KSS1 senes (the latter pair are distinct and functionally redundant). Accordingly, phosphorylation and/or activation of members of this kinase cascade can be detected and used to quantitate receptor engagement. Phosphotyrosine specific antibodies are available to measure increases in tyrosine phosphorylation and phospho-specific antibodies are commercially available (New England Biolabs, Beverly, MA).

In yet another embodiment, the signal transduction pathway of the LTM protein upregulates expression or otherwise activates an enzyme which is capable of cleaving a substrate which can be added to the cell. The signal can be detected by using a detectable substrate, in which case lose of the substrate signal is monitored, or altenatively, by using a substrate which produces a detectable product. In preferred embodiments, the conversion of the substrate to product by the activated enzyme produces a detectable change in optical characteristics of the test cell, e.g., the substrate and/or product is chromogenically or fluorogenically active. In an illustrative embodiment the, signal transduction pathway causes a change in the activity of a proteolytic enzyme, altering the rate at which it cleaves a substrate peptide (or simply activates the enzyme towards the substrate). The peptide includes a fluorogenic donor radical, e.g., a fluorescence emitting radical, and an acceptor radical, e.g., an aromatic radical which absorbs the fluorescence energy of the fluorogenic donor radical when the acceptor radical and the fluorogenic donor radical are covalently held in close proximity. See, for example, USSN 5,527,681, 5,506,115, 5,429,766, 5,424,186, and 5,316,691; and Capobianco et al. (1992) *Anal Biochem* 204:96-102. For example, the substrate

peptide has a fluorescence donor group such as 1-aminobenzoic acid (anthranilic acid or ABZ) or aminomethylcoumarin (AMC) located at one position on the peptide and a fluorescence quencher group, such as lucifer yellow, methyl red or nitrobenzo-2-oxo-1,3-diazole (NBD), at a different position near the distal end of the peptide. A cleavage site for the activated enzyme will be diposed between each of the sites for the donor and acceptor groups. The intramolecular resonance energy transfer from the fluorescence donor molecule to the quencher will quench the fluorescence of the donor molecule when the two are sufficiently proximate in space, e.g., when the peptide is intact. Upon cleavage of the peptide, however, the quencher is separated from the donor group, leaving behind a fluorescent fragment. Thus, activation of the enzyme results in cleavage of the detection peptide, and dequenching of the fluorescent group.

In still other embodiments, the detectable signal can be produced by use of enzymes or chromogenic/fluorscent probes whose activities are dependent on the concentration of a second messanger, e.g., such as calcium, hydrolysis products of inositol phosphate, cAMP, etc. For example , the mobilization of intracellular calcium or the influx of calcium from outside the cell can be measured using standard techniques. The choice of the appropriate calcium indicator, fluorescent, bioluminescent, metallochromic, or Ca++-sensitive microelectrodes depends on the cell type and the magnitude and time constant of the event under study (Borle (1990) *Environ Health Perspect* 84:45-56). As an exemplary method of Ca++ detection, cells could be loaded with the Ca++sensitive fluorescent dye fura-2 or indo-1, using standard methods, and any change in Ca++ measured using a fluorometer.

As certain embodiments described above suggest, in addition to directly measuring second messenger production, the signal transduction activity of a receptor or ion channel pathway can be measured by detection of a transcription product, e.g., by detecting receptor/channel-mediated transcriptional activation (or repression) of a gene(s). Detection of the transcription product includes detecting the gene transcript, detecting the product directly (e.g., by immunoassay) or detecting an activity of the protein (e.g., such as an enzymatic activity or chromogenic/fluorogenic activity); each of which is generally referred to herein as a means for detecting expression of the indicator gene. The indicator gene may be an unmodified endogenous gene of the host cell, a

modified endogenous gene, or a part of a completely heterologous construct, e.g., as part of a reporter gene construct.

In one embodiment, the indicator gene is an unmodified endogenous gene. In certain instances, it may be desirable to increase the level of transcriptional activation of the endogenous indicator gene by the signal pathway in order to, for example, improve the signal-to-noise of the test system, or to adjust the level of response to a level suitable for a particular detection technique. In one embodiment, the transcriptional activation ability of the signal pathway can be amplified by the overexpression of one or more of the proteins involved in the intracellular signal cascade, particularly enzymes involved in the pathway. For example, increased expression of Jun kinases (JNKs) can potentiate the level of transcriptional activation by a signal in an MEK/MEKK pathway. This approach can, of course, also be used to potentiate the level of transcription of a heterologous reporter gene as well.

In other embodiments, the sensitivity of an endogenous indicator gene can be enhanced by manipulating the promoter sequence at the natural locus for the indicator gene. Such manipulation may range from point mutations to the endogenous regulatory elements to gross replacement of all or substantial portions of the regulatory elements. In general, manipulation of the genomic sequence for the indicator gene can be carried out using techniques known in the art, including homologous recombination.

In another exemplary embodiment, the promoter (or other transcriptional regulatory sequences) of the endogenous gene can be "switched out" with a heterologous promoter sequence, e.g., to form a chimeric gene at the indicator gene locus. Again, using such techniques as homologous recombination, the regulatory sequence can be so altered at the genomic locus of the indicator gene.

In still another embodiment, a heterologous reporter gene construct can be used to provide the function of an indicator gene. Reporter gene constructs are prepared by operatively linking a reporter gene with at least one transcriptional regulatory element. If only one transcriptional regulatory element is included it must be a regulatable promoter, At least one the selected transcriptional regulatory elements must be indirectly or directly regulated by the activity of the selected cell-surface receptor whereby activity of the receptor can be monitored via transcription of the reporter genes.

Many reporter genes and transcriptional regulatory elements are known to those of skill in the art and others may be identified or synthesized by methods known to those of skill in the art.

g. Exemplary Screening and Selection Assays: Reporter Genes

Examples of reporter genes include, but are not limited to CAT (chloramphenicol acetyl transferase) (Alton and Vapnek (1979), Nature 282: 864-869) luciferase, and other enzyme detection systems, such as beta-galactosidase; firefly luciferase (deWet et al. (1987), Mol. Cell. Biol. 7:725-737); bacterial luciferase (Engebrecht and Silverman (1984), PNAS 1: 4154-4158; Baldwin et al. (1984), Biochemistry 23: 3663-3667); alkaline phosphatase (Toh et al. (1989) Eur. J. Biochem. 182: 231-238, Hall et al. (1983) J. Mol. Appl. Gen. 2: 101), human placental secreted alkaline phosphatase (Cullen and Malim (1992) Methods in Enzymol. 216:362-368).

Transcriptional control elements for use in the reporter gene constructs, or for modifying the genomic locus of an indicator gene include, but are not limited to, promoters, enhancers, and repressor and activator binding sites. Suitable transcriptional regulatory elements may be derived from the transcriptional regulatory regions of genes whose expression is rapidly induced, generally within minutes, of contact between the cell surface protein and the effector protein that modulates the activity of the cell surface protein. Examples of such genes include, but are not limited to, the immediate early genes (see, Sheng et al. (1990) Neuron 4: 477-485), such as c-fos. Immediate early genes are genes that are rapidly induced upon binding of a ligand to a cell surface protein. The transcriptional control elements that are preferred for use in the gene constructs include transcriptional control elements from immediate early genes, elements derived from other genes that exhibit some or all of the characteristics of the immediate early genes, or synthetic elements that are constructed such that genes in operative linkage therewith exhibit such characteristics. The characteristics of preferred genes from which the transcriptional control elements are derived include, but are not limited to, low or undetectable expression in quiescent cells, rapid induction at the transcriptional level within minutes of extracellular simulation, induction that is transient and independent of new protein synthesis, subsequent shut-off of transcription requires new protein synthesis, and mRNAs transcribed from these genes have a short half-life. It is not necessary for all of these properties to be present.

Other promoters and transcriptional control elements, in addition to those described above, include the vasoactive intestinal peptide (VIP) gene promoter (cAMP responsive; Fink et al. (1988), Proc. Natl. Acad. Sci. 85:6662-6666); the somatostatin gene promoter (cAMP responsive; Montminy et al. (1986), Proc. Natl. Acad. Sci. 8.3:6682-6686); the proenkephalin promoter (responsive to cAMP, nicotinic agonists, and phorbol esters; Comb et al. (1986), Nature 323:353-356); the phosphoenolpyruvate carboxy-kinase gene promoter (cAMP responsive; Short et al. (1986), J. Biol. Chem. 261:9721-9726); the NGFI-A gene promoter (responsive to NGF, cAMP, and serum; Changelian et al. (1989). Proc. Natl. Acad. Sci. 86:377-381); and others that may be known to or prepared by those of skill in the art.

In the case of receptors which modulate cyclic AMP, a transcriptional based readout can be constructed using the cyclic AMP response element binding protein, CREB, which is a transcription factor whose activity is regulated by phosphorylation at a particular serine (S133). When this serine residue is phosphorylated, CREB binds to a recognition sequence known as a CRE (cAMP Responsive Element) found to the 5' of promotors known to be responsive to elevated cAMP levels. Upon binding of phosphorylated CREB to a CRE, transcription from this promoter is increased.

Phosphorylation of CREB is seen in response to both increased cAMP levels and increased intracellular Ca levels. Increased cAMP levels result in activation of PKA, which in turn phosphorylates CREB and leads to binding to CRE and transcriptional activation. Increased intracellular calcium levels results in activation of calcium/calmodulin responsive kinase IV (CaM kinase IV). Phosphorylation of CREB by CaM kinase IV is effectively the same as phosphorylation of CREB by PKA, and results in transcriptional activation of CRE containing promotors. Activation of extracellular signal-related protein kinase (ERK) and Rsk2 by also leads to the phosphorylation and transactivation of CREB. Impey et al. (1998) Neuron 21:869-883.

Therefore, a transcriptional-based readout can be constructed in cells containing a reporter gene whose expression is driven by a basal promoter containing one or more CRE. Changes in the intracellular concentration of Ca^{++} (a result of alterations in the activity of the receptor upon engagement with a ligand) will result in changes in the level of expression of the reporter gene if: a) CREB is also co-expressed in the cell, and b) either the endogenous yeast CaM kinase will phosphorylate CREB in response to

increases in calcium or if an exogenously expressed CaM kinase IV is present in the same cell. In other words, stimulation of PLC activity will result in phosphorylation of CREB and increased transcription from the CRE-construct, while inhibition of PLC activity will result in decreased transcription from the CRE-responsive construct.

As described in Bonni et al. (1993) *Science* 262:1575-1579, the observation that CNTF treatment of SK-N-MC cells leads to the enhanced interaction of STAT/p91 and STAT related proteins with specific DNA sequences suggested that these proteins might be key regulators of changes in gene expression that are triggered by CNTF. Consistent with this possibility is the finding that DNA sequence elements similar to the consensus DNA sequence required for STAT/p91 binding are present upstream of a number of genes previously found to be induced by CNTF (e.g., Human c-fos, Mouse c-fos, Mouse tis11, Rat junB, Rat SOD-1, and CNTF). Those authors demonstrated the ability of STAT/p91-binding sites to confer CNTF responsiveness to a non-responsive reporter gene. Accordingly, a reporter construct for use in the present invention for detecting signal transduction through STAT proteins, such as from cytokine receptors, can be generated by using -71 to +109 of the mouse c-fos gene fused to the bacterial chloramphenicol acetyltransferase gene (-71fosCAT) or other detectable marker gene. Induction by a cytokine receptor induces the tyrosine phosphorylation of STAT and STAT-related proteins, with subsequent translocation and binding of these proteins to the STAT-RE. This then leads to activation of transcription of genes containing this DNA element within their promoters.

In preferred embodiments, the reporter gene is a gene whose expression causes a phenotypic change which is screenable or selectable. If the change is selectable, the phenotypic change creates a difference in the growth or survival rate between cells which express the reporter gene and those which do not. If the change is screenable, the phenotype change creates a difference in some detectable characteristic of the cells, by which the cells which express the marker may be distinguished from those which do not. Selection is preferable to screening in that it can provide a means for amplifying from the cell culture those cells which express a test polypeptide which is a receptor effector.

The marker gene is coupled to the receptor signaling pathway so that expression of the marker gene is dependent on activation of the receptor. This coupling may be achieved by operably linking the marker gene to a receptor-responsive promoter. The

term "receptor-responsive promoter" indicates a promoter which is regulated by some product of the target receptor's signal transduction pathway.

Alternatively, the promoter may be one which is repressed by the receptor pathway, thereby preventing expression of a product which is deleterious to the cell. With a receptor repressed promoter, one screens for agonists by linking the promoter to a deleterious gene, and for antagonists, by linking it to a beneficial gene. Repression may be achieved by operably linking a receptor- induced promoter to a gene encoding mRNA which is antisense to at least a portion of the mRNA encoded by the marker gene (whether in the coding or flanking regions), so as to inhibit translation of that mRNA. Repression may also be obtained by linking a receptor-induced promoter to a gene encoding a DNA binding repressor protein, and incorporating a suitable operator site into the promoter or other suitable region of the marker gene.

h. Exemplary emodiments: Host Cells

Suitable host cells for generating the subject assay include prokaryotes, yeast, or higher eukaryotic cells, especially mammalian cells. Prokaryotes include gram negative or gram positive organisms. Examples of suitable mammalian host cell lines include the COS-7 line of monkey kidney cells (ATCC CRL 1651) (Gluzman (1981) Cell 23:175) CV-1 cells (ATCC CCL 70), L cells, C127, 3T3, Chinese hamster ovary (CHO), HeLa and BHK cell lines. It will be understood that to achieve selection or screening, the host cell must have an appropriate phenotype.

If yeast cells are used, the yeast may be of any species which are cultivable and in which an exogenous receptor can be made to engage the appropriate signal transduction machinery of the host cell. Suitable species include *Kluyverei lactis, Schizosaccharomyces pombe*, and *Ustilaqo maydis; Saccharomyces cerevisiae* is preferred. Other yeast which can be used in practicing the present invention are *Neurospora crassa, Aspergillus niger, Aspergillus nidulans, Pichia pastoris, Candida tropicalis, and Hansenula polymorpha.* The term "yeast", as used herein, includes not only yeast in a strictly taxonomic sense, i.e., unicellular organisms, but also yeast-like multicellular fungi or filamentous fungi.

The choice of appropriate host cell will also be influenced by the choice of detection signal. For instance, reporter constructs, as described below, can provide a selectable or screenable trait upon transcriptional activation (or inactivation) in response

to a signal transduction pathway coupled to LTM protein of interest. The reporter gene may be an unmodified gene already in the host cell pathway, such as the genes responsible for growth arrest in yeast. It may be a host cell gene that has been operably linked to a "receptor-responsive" promoter. Alternatively, it may be a heterologous gene (e.g., a "reporter gene construct") that has been so linked. Suitable genes and promoters are discussed below. In other embodiments, second messenger generation can be measured directly in the detection step, such as mobilization of intracellular calcium or phospholipid metabolism are quantitated. In yet other embodiments indicator genes can be used to detect receptor-mediated signaling.

E. Use of Animal Models for Validating Drug Leads

Compounds which are identified as active in the above-described assays can be further assessed for *in vivo* efficacy in regulating LTM in a variety of animal systems.

In one embodment, such compounds can be tested in an animal model for studying fornix-mediated memory consolidation, e.g., an animal which as been prepared by introducing lesions to the formix structure. As we have previously described (Taubenfeld et al. (1999) Nat Neurosci 2:309), animals with fornix lesions learn the inhibitory avoidance and display memory at control levels for up to 6 hours, however, by 24 hours they exhibit amnesia.

In general, the subject method utilizes an animal which has been manipulated to create at least partial disruption of fornix-mediated signalling to the hippocampus, the disruption affecting memory consolidation and learned behavior in the animal. The animal is conditioned with a learning or memory regimen which results in learned behavior in the mammal in the absence of the fornix lesion. Test agents are administered to the animal in order assess their effects on memory consolidation. An increase in learned behavior, relative to the absence of the test agent, indicates that the test agent enhances memory consolidation.

In the methods of the present invention, retention of the learned behavior can be determined, for example, after at least about 12- 24 hours, 14-22 hours, 16-20 hours and or 18-19 hours after completion of the learning phase to determine whether the agent promotes memory consolidation. In a particular embodiment, retention of the learned behavior can be determined 24 hours after completion of the learning phase.

In the methods of the present invention, the lesion mammal can have a lesion of the fornix or a related brain structure that disrupts memory consolidation (e.g., perirhinal cortex, amygdala, medial septal nucleus, locus coeruleus, hippocampus, mammallary bodies). Lesions in the mammal can be produced by mechanical or chemical disruption. For example, the fornix lesion can be caused by surgical ablation, electrolytic, neurotoxic and other chemical ablation techniques, or reversible inactivation such as by injection of an anesthetic, e.g., tetrodooxin or lidocaine, to temporarily arrest activity in the fornix.

To further illustrate, fimbrio-fornix (rodents) and fornix (primates) lesions can be created by stereotatic ablation. In particular, neurons of the fornix structure are axotomized, e.g., by transection or aspiration (suction) ablation. A complete transection of the fornix disrupts cholinergic and GABAergic function and electrical activity, and induces morphological reorganization in the hippocampal formation. In general, the fornix transection utilized in the subject method will not disconnect the parahippocampal region from the neocortex. In those embodiments, the fornix transection will not disrupt functions that can be carried out by the parahippocampal region independent of processing by the hippocampal formation, and hence would not be expected to produce the full-blown amnesia seen following more complete hippocampal system damage.

In one embodiment, the animal can be a rat. Briefly, the animals are anesthetized, e.g., with intraperitoneal injections of a ketamine-xylazine mixture and positioned in a Kopf stereotoxic instrument. A sagittal incision is made in the scalp and a craniotomy is performed extending 2.0 mm posterior and 3.0 mm lateral from Bregma. An aspirative device, e.g., with a 20 gauge tip, is mounted to a stereotaxic frame (Kopf Instruments) and fimbria-fornix is aspirated by placing the suction tip at the correct sterotaxic location in the animals brain. Unilateral aspirative lesions are made by suction through the cingulate cortex, completely transecting the fimbria fornix unilaterally, and (optionally) removing the dorsal tip of the hippocampus as well as the overlying cingulate cortex to inflict a partial denervation on the hippocampus target. See also, Gage et al., (1983) Brain Res. 268:27 and Gage et al. (1986) Neuroscience 19:241.

In another exemplary embodiment, the animal can be a monkey. The animals can be anesthetized, e.g., with isoflurane (1.5-2.0%). Following pretreatment with mannitol (0.25 g/kg, iv), unilateral transections of the left fornix an be performed, such

as described by Kordower et al. (1990) J. Comp. Neurol., 298:443. Briefly, a surgical drill is used to create a parasagittal bone flap which exposes the frontal superior sagittal sinus. The dura is retracted and a self-retaining retractor is used to expose the interhemispheric fissure. The corpus callosum is longitudinally incised. At the level of the foramen of Monro, the fornix is easily visualized as a discrete 2-3 mm wide white fiber bundle. The fornix can be initially transected using a ball dissector. The cut ends of the fornix can then be suctioned to ensure completeness of the lesion.

In still other illustrative embodiments, the fornix lesion can be created by excitotoxically, or by other chemical means, inhibiting or ablating fornix neurons, or the cells of the hippocampus which are innervated by fornix neurons. In certain preferred embodiments, the fornix lesion is generated by selective disruption of particular neuronal types, such as fornix cholinergic, GABAergic and/or serotonergic neurons, and in certain embodiments, particular morphological subtypes within such neuron types. For instance, selective ablation of serotonergic neurons can be accomplished by treatment of the fornix structure with methamphetamines, such as d-methamphetamine (d-MA), methylenedioxyamphetamine (MDA) and methylenedioxymethamphetamine (MDMA), and 5,7-dihydroxytryptamine (5,7-DHT). The afferant fornix signals to the hippocampus due to cholinergic neurons can be ablated by atropine blockade. Another means for ablation of the cholinergic neurons is the use of 192IgG-saporin (192IgG-sap), e.g., intraventricularly injection into the fornix and hippocampus. In other embodiments, the agents such as 6-OHDA and ibotenic acid can be used to selectively destroy fornix dopamine neurons as part of the ablative regimen. Other exemplary agents which may used to create fornix lesions include N-methyl-D-aspartate (NMDA), quinolinic acid, and methylazoxymethanol.

There are a variety of test for cognitive function, especially learning and memory testing, which can be carried our using the lesioned and normal animals. Learning and/or memory tests include, for example, inhibitory avoidance, contextual fear conditioning, visual delay non-match to sample, spatial delay non-match to sample, visual discrimination, Barnes circular maze, Morris water maze and Radial arm maze tests.

An exemplary inhibitory avoidance test utilizes an apparatus that consists of a lit chamber that can be separated from a dark chamber by a sliding door. At training, the

animal is placed in the lit chamber for some period of time, then the door is opened. the animal moves to the dark chamber after a short delay-the latency, that is recorded. Upon entry into the dark chamber, the door is shut closed and a footshock is delivered. Retention of the experience is determined after various time intervals, e.g., 24 or 48 hours, by repeating the test and recording the latency. The protocol is one of many variants of the inhibitory avoidance procedures (for review, see Rush (1988) Behav Neural Biol 50:255).

An exemplary maze testing embodiment is the water maze working memory test. In general, the method utilizes an apparatus which consists of a circular water tank. The water in the tank is made cloudy by the addition of milk powder. A clear plexiglass platform, supported by a movable stand rest on the bottom of the tank, is submerged just below the water surface. Normally a swimming rat cannot perceive the location of the platform but it may recall it from a previous experience and training, unless it suffers from some memory impairment. The time taken to locate the platform is measured and referred to as the latency. During the experiment, all orientational cues such as ceiling lights etc. remain unchanged. Longer latencies are generally observed with rats with some impairment to their memory.

Another memory test includes the eyeblink conditioning test, which involves the administration of white noise or steady tone that preceeds a mild air puff which stimulates the subject's eyeblink.

Still another memory test which can be used is fear conditioning, e.g., either "cued" and "contextual" fear conditioning. In one embodiment, a freeze monitor administers a sequence of stimuli (sounds, shock) and then records a series of latencies measuring the recovery from shock induced freezing of the animal.

Another memory test for the lesioned animals is a holeboard test, which utilizes a rotating holeboard apparatus containing (four) open holes arranged in a 4-corner configuration in the floor of the test enclosure. A mouse is trained to poke its head into a hole and retrieve a food reward from a "baited" hole which contains a reward on every trial. There is a food reward (e.g. Froot Loop) in every exposed hole which is made inaccessible by being placed under a screen. The screen allows the odor of the reward to emanate from the hole, but does not allow access to the reinforcer. When an individual hole is baited, a small piece of Froot Loop is placed on top of the screen, where it is

accessible. The entire apparatus rests on a turntable so that it may be rotated easily to eliminate reliance on proximal (e.g. olfactory) cues. A start tube is placed in the center of the apparatus. The subject is released from the tube and allowed to explore for the baited ("correct") hole.

F. Pharmaceutical Preparations of Identified Agents

After identifying certain test compounds in the subject assay ,e.g., as potentiators or inhibitors of memory consolidation, the practioner of the subject assay will continue to test the efficacy and specificity of the selected compounds both *in vitro* and *in vivo*. Whether for subsequent *in vivo* testing, or for administration to an animal as an approved drug, agents identified in the subject assay can be formulated in pharmaceutically acceptable excipients for *in vivo* administration to an animal, preferably a human.

The compounds selected in the subject assay, or a pharmaceutically acceptable salt thereof, may accordingly be formulated for administration with a biologically acceptable medium, such as water, buffered saline, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol and the like) or suitable mixtures thereof. The optimum concentration of the active ingredient(s) in the chosen medium can be determined empirically, according to procedures well known to medicinal chemists. As used herein, "biologically acceptable medium" includes any and all solvents, dispersion media, and the like which may be appropriate for the desired route of administration of the pharmaceutical preparation. The use of such media for pharmaceutically active substances is known in the art. Except insofar as any conventional media or agent is incompatible with the activity of the compound, its use in the pharmaceutical preparation of the invention is contemplated. Suitable vehicles and their formulation inclusive of other proteins are described, for example, in the book *Remington's Pharmaceutical Sciences* (Remington's Pharmaceutical Sciences. Mack Publishing Company, Easton, Pa., USA 1985). These vehicles include injectable "deposit formulations". Based on the above, such pharmaceutical formulations include, although not exclusively, solutions or freeze-dried powders of the compound in association with one or more pharmaceutically acceptable vehicles or diluents, and contained in buffered media at a suitable pH and isosmotic with physiological fluids. In preferred embodiment, the compound can be disposed in a sterile preparation for topical and/or systemic administration. In the case of freeze-dried preparations, supporting excipients

such as, but not exclusively, mannitol or glycine may be used and appropriate buffered solutions of the desired volume will be provided so as to obtain adequate isotonic buffered solutions of the desired pH. Similar solutions may also be used for the pharmaceutical compositions of compounds in isotonic solutions of the desired volume and include, but not exclusively, the use of buffered saline solutions with phosphate or citrate at suitable concentrations so as to obtain at all times isotonic pharmaceutical preparations of the desired pH, (for example, neutral pH).

In certain embodiment, the pharmaceutical of the present invention is a gene delivery system for gene therapy with a therapeutic LTM gene. Such gene therapy systems can be introduced into a patient by any of a number of methods, each of which is familiar in the art. For instance, a pharmaceutical preparation of the gene delivery system can be introduced systemically, e.g. by intravenous injection, and specific transduction of the protein in the target cells occurs predominantly from specificity of transfection provided by the gene delivery vehicle, cell-type or tissue-type expression due to the transcriptional regulatory sequences controlling expression of the receptor gene, or a combination thereof. In other embodiments, initial delivery of the recombinant gene is more limited with introduction into the animal being quite localized. For example, the gene delivery vehicle can be introduced by catheter (see U.S. Pat. No. *5,328,470*) or by stereotactic injection (e.g. Chen et al. (1994) Proc. Natl. Acad. Sci. USA 91: 3054-3057).

The pharmaceutical preparation of the gene therapy construct can consist essentially of the gene delivery system in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery system can be produced intact from recombinant cells, e.g. retroviral vectors, the pharmaceutical preparation can comprise one or more cells which produce the gene delivery system.

F. Methods of Treatment

In various embodiments, the present invention contemplates modes of treatment and prophylaxis which utilitize one or more of the subject LTM genes (e.g., by gene therapy) or antisense constructs thereto, the LTM proteins (e.g., for protein therapy) or peptidomimetics thereof, or compounds identified in the subject drug screening assays. These agents may be useful for altering (increasing or decreasing) the occurrence of

learning and/or memory defects in an organism, and thus, altering the learning ability and/or memory capacity of the organism. In other embodiments, the preparations of the present invention can be used simply to enhance normal memory function.

Memory disorders which can be treated according to the present invention may have a number of origins: a functional mechanism (anxiety, depression), physiological aging (age-associated memory impairment), drugs, or anatomical lesions (dementia). Indications for which such preparations may be useful include learning disabilities, memory impairment, e.g., due to toxicant exposure, brain injury, age, schizophrenia, epilepsy, mental retardation in children and senile dementia, including Alzheimer's disease.

In certain embodiments, the invention contemplates the treatment of amnesia. Amnesias are described as specific defects in declarative memory. Faithful encoding of memory requires a registration, rehearsal, and retention of information. The first two elements appear to involve the hippocampus and medial temporal lobe structures. The retention or storage appears to involve the heteromodal association areas. Amnesia can be experienced as a loss of stored memory or an inability to form new memories. The loss of stored memories is known as retrograde amnesia. The inability to form new memories is known as anterograde amnesia.

Complaints of memory problems are common. Poor concentration, poor arousal and poor attention all may disrupt the memory process to a degree. The subjective complaint of memory problems therefore must be distinguished from true amnesias. This is usually done at the bedside in a more gross evaluation and through specific neuropsychological test. Defects in visual and verbal memory can be separated through such test. In amnesias there is by definition a preservation of other mental capacities such as logic. The neurobiologic theory of memory described above would predict that amnesias would have relatively few pathobiologic variations. Clinically the problem of amnesias often presents as a result of a sudden illness in an otherwise healthy person.

Exemplary forms of amnesias which may be treated by the subject method include, amensias of short duration, alcoholic blackouts, Wernicke-Korsakoff's (early), partial complex seizures, transient global amnesia, those which are medication related, such as triazolam (Halcion), basilar artery migraines. The subject method may also be

used to treat amensias of longer duration, such as post concussive or as the result of Herpes simplex encephalitis .

(i) Effective Dose

Toxicity and therapeutic efficacy of compounds to be used in the treatment methods of the present invention can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining The LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50. Compounds which exhibit large therapeutic indices are preferred. While compounds that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC50 (i.e., the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

G. Diagnostic and Prognostic Assays

The present method also provides a method for determining if a subject is at risk for a disorder characterized deterioration of memory consolidation. In preferred embodiments, the methods can be characterized as comprising detecting, in a sample of cells from the subject, the presence or absence of a genetic lesion characterized by at least one of (i) an alteration affecting the integrity of a gene encoding an LTM protein, or (ii) the mis-expression of the LTM gene. To illustrate, such genetic lesions can be

detected by ascertaining the existence of at least one of (i) a deletion of one or more nucleotides from an LTM gene, (ii) an addition of one or more nuclectides to an LTM gene, (iii) a substitution of one or more nucleotides of an LTM gene, (iv) a gross chromosomal rearrangement of an LTM gene, (v) a gross alteration in the level of a messenger RNA transcript of an LTM gene, (vii) aberrant modification of an LTM gene, such as of the methylation pattern of the genomic DNA, (vii) the presence of a non-wild type splicing pattern of a messenger RNA transcript of an LTM gene, (viii) a non-wild type level of an LTM-protein, (ix) allelic loss of an LTM gene, and (x) inappropriate post-translational modification of an LTM-protein. As set out below, the present invention provides a large number of assay techniques for detecting lesions in an LTM gene, and importantly, provides the ability to discern between different molecular causes underlying a disorder.

In an exemplary embodiment, there is provided a nucleic acid composition comprising a (purified) oligonucleotide probe including a region of nucleotide sequence which is capable of hybridizing to a sense or antisense sequence of an LTM gene, such as represented by any of SEQ ID NOs: 1, 3, 5, 7, 9 or 11, or naturally occurring mutants thereof, or 5' or 3' flanking sequences or intronic sequences naturally associated with the subject LTM genes or naturally occurring mutants thereof. The nucleic acid of a cell is rendered accessible for hybridization, the probe is exposed to nucleic acid of the sample, and the hybridization of the probe to the sample nucleic acid is detected. Such techniques can be used to detect lesions at either the genomic or mRNA level, including deletions, substitutions, etc., as well as to determine mRNA transcript levels.

In preferred embodiments, the method can be generally characterized as comprising detecting, in a sample of cells from the subject, the presence or absence of a genetic lesion characterized by an alteration affecting the integrity of an LTM gene. To illustrate, such genetic lesions can be detected by ascertaining the existence of at least one of (i) a deletion of one or more nucleotides from an LTM gene, (ii) an addition of one or more nucleotides to an LTM gene, (iii) a substitution of one or more nucleotides of an LTM gene, and (iv) the presence of a non-wild type splicing pattern of a messenger RNA transcript of an LTM gene. As set out below, the present invention provides a large number of assay techniques for detecting lesions in LTM genes.

In certain embodiments, detection of the lesion comprises utilizing the probe/primer in a polymerase chain reaction (PCR) (see, e.g. U.S. Pat. Nos. *4,683,195* and *4,683,202*), such as anchor PCR or RACE PCR, or, alternatively, in a ligation chain reaction (LCR) (see, e.g., Landegran et al. (1988) Science 241:1077-1080; and Nakazawa et al. (1994) PNAS 91:360-364), the latter of which can be particularly useful for detecting point mutations in the LTM gene (see Abravaya et al. (1995) Nuc Acid Res 23:675-682). In a merely illustrative embodiment, the method includes the steps of (i) collecting a sample of cells from a patient, (ii) isolating nucleic acid (c .g., genomic, mRNA or both) from the cells of the sample, (iii) contacting the nucleic acid sample with one or more primers which specifically hybridize to an LTM gene under conditions such that hybridization and amplification of the LTM gene (if present) occurs, and (iv) detecting the presence or absence of an amplification product, or detecting the size of the amplification product and comparing the length to a control sample. It is anticipated that PCR and/or LCR may be desirable to use as a preliminary amplification step in conjunction with any of the techniques used for detecting mutations described herein.

Another embodiment of the invention provides for a nucleic acid composition comprising a (purified) oligonucleotide probe including a region of nucleotide sequence which is capable of hybridizing to a sense or antisense sequence of an LTM gene, or naturally occurring mutants thereof, or 5' or 3' flanking sequences or intronic sequences naturally associated with the subject LTM genes or naturally occurring mutants thereof. The nucleic acid of a cell is rendered accessible for hybridization, the probe is exposed to nucleic acid of the sample, and the hybridization of the probe to the sample nucleic acid is detected. Such techniques can be used to detect lesions at either the genomic or mRNA level, including deletions, substitutions, etc., as well as to determine mRNA transcript levels. Such oligonucleotide probes can be used for both predictive and therapeutic evaluation of allelic mutations which might be manifest in, for example, deterioration in memeory consolidation.

The methods described herein may be performed, for example, by utilizing pre-packaged diagnostic kits comprising at least one probe nucleic acid or antibody reagent described herein, which may be conveniently used, e.g., in clinical settings to diagnose patients exhibiting symptoms or family history of a disease or illness involving memory or an LTM gene.

Antibodies directed against wild type or mutant LTM proteins, which are discussed, above, may also be used in disease diagnostics and prognostics. Such diagnostic methods, may be used to detect abnormalities in the level of LTM protein expression, or abnormalities in the structure and/or tissue, cellular, or subcellular location of LTM protein. Structural differences may include, for example, differences in the size, electronegativity, or antigenicity of the mutant LTM protein relative to the normal LTM protein. Protein from the tissue or cell type to be analyzed may easily be detected or isolated using techniques which are well known to one of skill in the art, including but not limited to western blot analysis. For a detailed explanation of methods for carrying out western blot analysis, see Sambrook et al, 1989, supra, at Chapter 18. The protein detection and isolation methods employed herein may also be such as those described in Harlow and Lane, for example, (Harlow, E. and Lane, D., 1988, "Antibodies: A Laboratory Manual", Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.), which is incorporated herein by reference in its entirety.

This can be accomplished, for example, by immunofluorescence techniques employing a fluorescently labeled antibody (see below) coupled with light microscopic, flow cytometric, or fluorimetric detection. The antibodies (or fragments thereof) useful in the present invention may, additionally, be employed histologically, as in immunofluorescence or immunoelectron microscopy, for in situ detection of LTM proteins. In situ detection may be accomplished by removing a histological specimen from a patient, and applying thereto a labeled antibody of the present invention. The antibody (or fragment) is preferably applied by overlaying the labeled antibody (or fragment) onto a biological sample. Through the use of such a procedure, it is possible to determine not only the presence of the LTM protein, but also its distribution in the examined tissue. Using the present invention, one of ordinary skill will readily perceive that any of a wide variety of histological methods (such as staining procedures) can be modified in order to achieve such in situ detection.

Often a solid phase support or carrier is used as a support capable of binding an antigen or an antibody. Well-known supports or carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, gabbros, and magnetite. The nature of the carrier can be either soluble to some extent or insoluble for the purposes of the present invention. The support

material may have virtually any possible structural configuration so long as the coupled molecule is capable of binding to an antigen or antibody. Thus, the support configuration may be spherical, as in a bead, or cylindrical, as in the inside surface of a test tube, or the external surface of a rod. Alternatively, the surface may be flat such as a sheet, test strip, etc. Preferred supports include polystyrene beads. Those skilled in the art will know many other suitable carriers for binding antibody or antigen, or will be able to ascertain the same by use of routine experimentation.

One means for labeling an anti-LTM protein specific antibody is via linkage to an enzyme and use in an enzyme immunoassay (EIA) (Voller, "The Enzyme Linked Immunosorbent Assay (ELISA)", Diagnostic Horizons 2:1-7, 1978, Microbiological Associates Quarterly Publication, Walkersville, Md.; Voller, et al., J. Clin. Pathol. 31:507-520 (1978); Butler, Meth. Enzymol. 73:482-523 (1981); Maggio, (ed.) Enzyme Immunoassay, CRC Press, Boca Raton, Fla., 1980; Ishikawa, et al., (eds.) Enzyme Immunoassay, Kgaku Shoin, Tokyo, 1981). The enzyme which is bound to the antibody will react with an appropriate substrate, preferably a chromogenic substrate, in such a manner as to produce a chemical moiety which can be detected, for example, by spectrophotometric, fluorimetric or by visual means. Enzymes which can be used to detectably label the antibody include, but are not limited to, malate dehydrogenase, staphylococcal nuclease, delta-5-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate, dehydrogenase, triose phosphate isomerase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase and acetylcholinesterase. The detection can be accomplished by colorimetric methods which employ a chromogenic substrate for the enzyme. Detection may also be accomplished by visual comparison of the extent of enzymatic reaction of a substrate in comparison with similarly prepared standards.

Detection may also be accomplished using any of a variety of other immunoassays. For example, by radioactively labeling the antibodies or antibody fragments, it is possible to detect fingerprint gene wild type or mutant peptides through the use of a radioimmunoassay (RIA) (see, for example, Weintraub, B., Principles of Radioimmunoassays, Seventh Training Course on Radioligand Assay Techniques, The Endocrine Society, March, 1986, which is incorporated by reference herein). The

radioactive isotope can be detected by such means as the use of a gamma counter or a scintillation counter or by autoradiography.

It is also possible to label the antibody with a fluorescent compound. When the fluorescently labeled antibody is exposed to light of the proper wave length, its presence can then be detected due to fluorescence. Among the most commonly used fluorescent labeling compounds are fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine.

The antibody can also be detectably labeled using fluorescence emitting metals such as ^{152}Eu, or others of the lanthanide series. These metals can be attached to the antibody using such metal chelating groups as diethylenetriaminepentacetic acid (DTPA) or ethylenediaminetetraacetic acid (EDTA).

The antibody also can be detectably labeled by coupling it to a chemiluminescent compound. The presence of the chemiluminescent-tagged antibody is then determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of particularly useful chemiluminescent labeling compounds are luminol, isoluminol, theromatic acridinium ester, imidazole, acridinium salt and oxalate ester. Likewise, a bioluminescent compound may be used to label the antibody of the present invention. Bioluminescence is a type of chemiluminescence found in biological systems in, which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the presence of luminescence. Important bioluminescent compounds for purposes of labeling are luciferin, luciferase and aequorin.

Moreover, it will be understood that any of the above methods for detecting alterations in an LTM gene or gene product can be used to monitor the course of treatment or therapy.

H. Transgenic Animals

These systems may be used in a variety of applications. For example, the cell- and animal-based model systems may be used to further characterize the role of the subject LTM genes and proteins in memory. In addition, such assays may be utilized as part of screening strategies designed to identify compounds which are capable of ameliorating disease symptoms. Thus, the animal- and cell-based models may be used to

identify drugs, pharmaceuticals, therapies and interventions which may be effective in treating disease.

One aspect of the present invention concerns the use of a transgenic animal which is comprised of cells (of that animal) which contain a transgene of the present invention and which preferably (though optionally) express an exogenous LTM protein in one or more cells in the animal. An LTM transgene can encode the wild-type form of the protein, or can encode homologs thereof including both agonists and antagonists, as well as antisense constructs. In preferred embodiments, the expression of the transgene is restricted to specific subsets of cells, tissues or developmental stages utilizing, for example, cis-acting sequences that control expression in the desired pattern. In the present invention, such mosaic expression of an LTM protein can be essential for many forms of lineage analysis and can additionally provide a means to assess the effects of, for example, lack of LTM expression which might grossly alter development in small patches of tissue within an otherwise normal embryo. Toward this and, tissue-specific regulatory sequences and conditional regulatory sequences can be used to control expression of the transgene in certain spatial patterns. Moreover, temporal patterns of expression can be provided by, for example, conditional recombination systems or prokaryotic transcriptional regulatory sequences.

In an exemplary embodiment, the "transgenic non-human animals" of the invention are produced by introducing transgenes into the germline of the non-human animal. Embryonal target cells at various developmental stages can be used to introduce transgenes. Different methods are used depending on the stage of development of the embryonal target cell. The specific line(s) of any animal used to practice this invention are selected for general good health, good embryo yields, good pronuclear visibility in the embryo, and good reproductive fitness. In addition, the haplotype is a significant factor. For example, when transgenic mice are to be produced, strains such as C57BL/6 or FVB lines are often used (Jackson Laboratory, Bar Harbor, Me.). Preferred strains are those with $H\text{-}2^b$, $H\text{-}2^d$ or $H\text{-}2^q$ haplotypes such as C57BL/6 or DBA/1. The line(s) used to practice this invention may themselves be transgenics, and/or may be knockouts (i.e., obtained from animals which have one or more genes partially or (completely suppressed).

In one embodiment, gene targeting, which is a method of using homologous recombination to modify an animal's genome. can be used to introduce changes into cultured embryonic stem cells. By targeting an LTM gene of interest in ES cells, these changes can be introduced into the germlines of animals to generate chimeras. The gene targeting procedure is accomplished by introducing into tissue culture cells a DNA targeting construct that includes a segment homologous to a target LTM locus, and which also includes an intended sequence modification to the LTM genomic sequence (e.g., insertion, deletion, point mutation). The treated cells are then screened for accurate targeting to identify and isolate those which have been properly targeted.

Gene targeting in embryonic stem cells is in fact a scheme contemplated by the present invention as a means for disrupting an LTM gene function through the use of a targeting transgene construct designed to undergo homologous recombination with one or more LTM genomic sequences. The targeting constrict can be arranged so that, upon recombination with an element of an LTM gene, a positive selection marker is inserted into (or replaces) coding sequences of the targeted gene. The inserted sequence functionally disrupts the LTM gene, while also providing a positive selection trait. Exemplary LTM targeting constructs are described in more detail below.

The present invention is further illustrated by the following examples which should not be construed as limiting in any way. The contents of all cited references (including literature references, issued patents, published patent applications as cited throughout this application are hereby expressly incorporated by reference.

Exemplification

Example 1

Northern blot analysis reveals that several immediate-early genes are induced following IA training and that this training-related gene regulation is sensitive to fornix lesions.

In the invertebrate *Aplysia californica*, it has been demonstrated that the gene expression changes which underlie memory formation begin with the induction of regulatory immediate-early genes (IEGs). Specifically, induction of ApC/EBP, a member of the CCAAT enhancer binding protein (C/EBP) transcription factor family, is essential for long-term synaptic plasticity that underlies memory formation in *Aplysia*

(Alberini, 1994). Based on these data, we proposed that memory-inducing stimuli activate a gene cascade, where CREB regulates the expression of regulatory IEGs which, in turn, regulate the expression of more downstream target genes required for long-term memory (Alberini, 1994). This model accounts for why gene expression seems to be required only for an early and brief time window. The essential gene expression appears to be brief because it corresponds to the critical time necessary for the expression of regulatory IEGs, which would be the rate-limiting step of the molecular cascade of events leading to long-term memory.

In mammals, changes in mRNA and protein levels of IEGs such as c-fos and zif268 have been widely investigated in memory and models of synaptic plasticity. Although still controversial (Campeau, 1991), particularly due to the lack of precise controls or quantitative measurements (the studies have been generally based on immunohistochemical analysis), both c-fos and zif268 have been reported to be up-regulated in several brain areas following different kinds of learning (rev. in Dragunow, 1996). Therefore, in our initial pilot experiments, we performed a Northern blot analysis of changes in hippocampal zif268 and c-fos mRNA levels following IA training, focusing on the temporal window suggested by induction of PCREB; i.e., 3, 6, 9, and 20 hrs after training. Three animals per timepoint were investigated, and the hybridizations were normalized using cyclophilin gene as a control probe. The same membrane was sequentially hybridized with all the probes. As shown in Fig. 1, we found that zif268 was induced in all the trained animals at 9 and 20 hr after training. On the contrary, c-fos did not show any evident change throughout the timecourse. Thus, the IEG response to IA training are selective with respect to the genes activated as well as the time following training.

Fig. 1. Time-course Northern blot analysis of zif268 and c-fos following IA training. Increase in zif268, but not c-fos mRNA is evident in all animals at 9 and 20 hr after training. These results showed that zif268 is induced in the hippocampus by IA. They also reveal *when* the induction of an IEG is detectable in the hippocampus following IA, namely at 9 and 20 hrs. On the basis of these data, as described below, we decided to perform our first array hybridization analysis using the RNA obtained from animals 9 hr after training.

Encouraged by these results with zif268, we subsequently hybridized the same blot using probes directed at additional transcription factors, including C/EBP β□ a member of the CCAAT enhancer binding protein family. We found that C/EBP β was induced following IA training during a time window similar to that of zif268 (Fig 2). This finding is extremely compelling, since it suggests the possibility that like CREB, members of the C/EBP family, might be evolutionarily conserved components of the gene cascade activated during memory formation.

To define whether the increase in C/EBP□β expression is related to IA memory rather than exploring a new environment, the IA trained animals were compared at each timepoint to *paired-control* animals that walked through the inhibitory avoidance apparatus without receiving a footshock in the dark chamber. Moreover, the level of hippocampal C/EBP β mRNA did not change in animals that received only the shock without the contextual learning and were sacrificed 9 and 20 hr later (not shown). This is not surprising since both learning (Fanselow, 1990) and an increase in PCREB (Taubenfeld et al. 1999) do not occur in animals that received the shock only. Therefore, C/EBP□β seems to be selectively induced by IA training.

We then began to address the question of whether the C/EBP β response that we found to be associated with memory is sensitive to fornix lesions. As shown in Fig 2, animals with lesions failed to show any change in the expression of C/EBPβ after IA training.

Figure 2. Time course Northern blot analysis of C/EBPβ and cyclophilin (control) mRNA following IA training in hippocampi of unoperated and fornix-lesion rats. Importantly, the level of hippocampal C/EBP□β in animals with fornix lesions does not differ from that of unoperated animals (compare 0 no shock and 0 no shoch fornix lesion in Figure 2). This finding clearly demonstrates that the fornix is specifically required for this gene response to IA training, and considerably strengthens the hypothesis that the changes we have observed are selectively related to memory consolidation.

Example 2

Array hybridization reveals new gene responses to IA training.

We believe that our model system lends itself particularly well to searching for genes differentially expressed in long-term memory. We have determined where and when to carry out the differential analysis in animals that have the ability to learn vs. those with memory impairment. We also know that learning induces a hippocampal gene response that is sensitive to fornix lesions.

The possibility of identifying differentially expressed genes is one of the most powerful approaches for understanding the gene pattern linked to a specific function. A multitude of techniques have become available in recent years to isolate differentially expressed genes and some have been successfully used in isolating genes involved in long-term memory (Cavallaro, 1997). The most advanced generation of these techniques is the hybridization of DNA arrays. The arrays consist of supports (nylon or glass) on which cDNA fragments have been immobilized systematically. A schematic representation of how this technique works is shown in Figure 3.

Figure 3. Broad scale expression profiling with cDNA expression arrays. Side by side hybridizations with cDNA probes prepared from two different RNA populations allow the simultaneous comparison of the expression levels of all the cDNAs on the array. (from Clontech user manual). Arrays can contain, in principle, all the cloned DNA sequences. We begun our analysis using a relatively small array, the AtlasTM rat cDNA expression array from Clontech (PaloAlto, Ca), which was the most complete commercially available array at that time. This array contained 588 genes isolated from rat brain and coding for a variety of molecules involved in the pathways that regulate brain function, such as signaling molecules, receptors, signal transduction proteins, extracellular proteins, structural molecules, molecules involved in synaptic transmission, and molecules involved in neural pathologies, including Alzheimer disease. The DNAs were fixed on a positively charged nylon membrane.

Since it is possible to detect changes in mRNA expression at 9 hr after IA training compared to controls, we began the search for differential gene expression in memory using array hybridization and compared animals that walked through the apparatus without receiving a shock (0 no shock) and were immediately sacrificed to animals that underwent IA training and were sacrificed 9 hr later. Hippocampi from 4

control (0 no shock) and 4 trained animals were dissected, and their RNAs were isolated and pooled. The pooled RNAs from control and trained animals were used to hybridize two identical membranes. The array hybridizations appeared very clean; only a few spots out of 588 carried on the membranes showed changes in their hybridization signal. Importantly, these spots that showed changes in intensity were surrounded by others that had very comparable hybridization signals. In all, 9 hr after IA training, we found changes in the concentration of approximately 15 transcripts, eight of which have been analyzed and confirmed by Northern analysis. Fig. 4 shows two examples of our results using the arrays. (To protect the confidentiality of the results, the names of some genes are not revealed. They are identified with numbers. However if this presents an obstacle for considering the present application, the identity of the genes will be disclosed.) As described in fig 4, we found an increase in a spot that corresponded to zif268. This confirmed that the array analysis is sensitive enough to detect changes we have observed with Northern blot hybridization. Another spot, here named gene #2 appeared to be clearly decreased.

Figure 4. Examples of changes in gene array hybridizations reflecting differential expression of mRNAs following IA training. Hippocampi of control rats are compared to hippocampi of rats trained and sacrificed 9 hr later. Note that on these arrays each sequence is spotted in duplicate. The array hybridization is a powerful method of screening. However, to prove that the transcripts identified with this technique indeed change their expression following IA learning, a Northern blot hybridization analysis of each identified sequence was necessary. The Northern blot, and not the array hybridization, ultimately defines whether a gene changes its expression after learning, therefore all the necessary controls were carried out with the Northern blot hybridization and not with array hybridization. The same membrane carrying the time-course analysis described above in Fig 4, used to analyze C/EBP β, was subsequently rehybridized to analyze the expression of gene #2 and of another gene that also appeared to decrease in the array hybridizations and that we called gene #3. As shown in Fig. 5, the Northern blot test confirmed that the levels of both transcripts #2 and 3 were strongly decreased at 9 and 20 hr post-training. No changes were observed at 3 and 6 hr after IA, and no significant changes were detected in paired control animals at any timepoint.

These results show that the regulation of genes #2 and #3 is selectively associated with IA training. As with C/EBPβ, the regulation of gene #2 that occurred with learning was abolished in fornix-lesioned animals, although the basal expression of the gene was not affected by lesioning. These data confirmed the IA memory formation is accompanied by regulation of gene expression that is induced by inputs passing through the fornix. Gene #3, on the other hand, appeared to be down-regulated with IA memory as well as in all animals with fornix lesions. From these data we conclude that fornix lesions produce changes in the regulation of certain genes within the hippocampus. This result is intriguing and suggests that the fornix may contribute to memory formation by modulating either constitutive or IA-induced gene expression.

Taken together, these data demonstrate that we are able to detect and analyze changes in gene expression after IA training. We have confirmed that some of these changes do not occur in the hippocampi of animals with lesions of the fornix which have impaired memory consolidation. We believe that these preliminary data show convincingly that our IA model is suitable for the analysis of gene expression changes in long-term memory.

We recently began to establish hybridization conditions of more complete arrays that have very recently become available from Research Genetics. These arrays contain 5,000 rat transcripts. Our goal is to carry out a systematic analysis of the rat cloned genes and identify which change their expression following training. To screen for genes whose expression change at different times after learning, as described in the Research Plan section, we plan to analyze several time windows after training and follow up with Northern blot analyses. Moreover, to define whether the gene cascade activated in IA is a general molecular mechanism of memory, we will analyze the expression of the identified genes in other forms of memory, including contextual fear conditioning and Morris water maze.

Example 3

The delayed and prolonged gene response during memory consolidation makes feasible detection of changes after the Morris water maze.

One hopes that the genes involved in long-term memory consolidation are conserved, not only evolutionarily, but also across different types of memory that utilize the same neural structures. We chose IA training originally because it could be used to

precisely define the time-course of gene activation. Our survey of PCREB changes in the brain led us to focus our attention on the hippocampus. The hippocampus is also essential for the expression of memory probed using the Morris water maze (Riedel, 1999). Water maze training was not our first choice for investigating the gene response during memory consolidation because it typically takes many trials delivered over several days. However, the data from IA training suggest that fairly prolonged gene responses can lag behind the initial training period for up to 24 hrs. We have therefore trained rats in the water maze to investigate how much memory is demonstrated 24 hrs after a single day of training.

The Morris water maze is a very reliable and widely used assay of hippocampal function. Rats with damage to any part of the hippocampal system including the hippocampal formation itself, the entorhinal cortex and fornix show severe deficits on this task (Hannenson, 1998; Sutherland, 1989). The water maze apparatus consists of a large, circular pool filled with water made opaque by the addition of white tempura paint. An escape platform is hidden just below the surface of the water, and the rat is required to swim to the hidden platform from different starting points around the edge of the pool. Normal rats can acquire this task quickly by using and remembering configurations of extramaze cues to navigate directly to the platform. Learning is reflected in shorter latencies to escape and decreases in the length of the path that the rat swims in order to reach the platform.

In the following experiment, control rats (n = 6) were trained on the water maze in order to determine how much information can be learned and remembered over a relatively brief training period. Each rat received eight trials a day for four consecutive days. As can be seen from Figure 8 control rats learned to swim directly to the platform very quickly, reaching asymptotic performance after three days of training. A substantial amount of learning was seen after just one day of training, as mean escape latency decreased from 39.88 seconds on day one to 16.81 seconds on day 2 (see also figure 8). These results indicate that a reasonably robust spatial memory is formed after one day of training on the water maze. This task, therefore, may be included in our analysis of gene expression changes in long-term memory.

Figure 6. Panel A. Mean escape latency for rats trained on the water maze. Rats received eight trials a day, for four consecutive days. Panel B. Mean escape latency on each of the eight trials of day one (trials 1-8) and day two (trials 9-16).

Example 4

Changes in gene expression detected by hybridizations of array.

In this project we will identify which genes, among approximately 5,000 transcripts including genes and expressed sequence tag (ESTs), are regulated in the hippocampus during IA memory formation. We will compare parallel hybridizations of identical arrays with cDNA probes obtained from hippocampal RNA of untrained and trained animals. The transcripts that will show a significant change in expression in the trained condition ws the untrained will be further analyzed by Northern blot, following the sequence of steps described above in 1 to 4.

The array hybridization screening for genes differentially expressed following IA training will be carried on arrays purchased from Research Genetics (Huntsville, AL). This company has recently released the most complete rat DNA array commercially available, the *rat GeneFilters microarrays*. These arrays contain over 5,000 spots, representing approximately 1,700 named rat genes as well as many rat ESTs that are considered similar to named genes in other organisms. Each spot on the membrane contains approximately 0.5 ng of insert DNA from a cDNA clone containing the 3' end of a gene. The insert cDNA has been denatured and UV-cross-linked to the positively charged membrane. The manufacturer provides a detailed protocol that insures that the hybridizations are carried out under conditions where most probes (cDNA from the experimental mRNAs) are not saturating the spots. However, as described below we will set up several hybridization conditions that will provide the highest probability of success for identifying genes regulated in memory. These arrays (nylon membranes) are similar to those we purchased from Clontech and successfully used to identify several genes, as described in Preliminary Studies. However, they contain a much greater number of transcripts. Finally, Research Genetics also provides a software analysis tool that, when used in conjunction with Gene Filters, allows for the comparison of gene expression from images produced on a phosphor imaging system. This system allows for normalization across multiple experiments and has a built-in database to facilitate archiving of both raw images and fitted data. To maximize the probability of detecting

changes in gene expression levels following IA training we will perform multiple array hybridizations.

We Claim:

1. A method for modulating long term memory consolidation in an animal comprising treating an animal with an agent that modulates the activity of one or more of zif 268, insulin-like growth factor, glutamate receptor 1 (GluR1), glutamate receptor 2 (GluR2), c/EBPβ and VGF.

2. A method for enhancing long term memory consolidation in an animal comprising treating an animal with an agent that modulates a signal transduction pathway of glutamate receptor 1 (GluR1) or glutamate receptor 2 (GluR2), which agent is a ligand for the GluR1 or GluR2 receptor.

3. A method for identifying an agent which modulates memory consolidation, comprising,
   (i) providing a reaction system for detecting the activity of a product encoded by a gene selected from the group consisting of of zif 268, insulin-like growth factor, glutamate receptor 1 (GluR1), glutamate receptor 2 (GluR2), c/EBPβ and VGF;
   (ii) contacting said system with a test compound; and
   (iii) determining if the test compound alters the activity of the gene product.

4. A method for identifying an agent which modulates memory consolidation, comprising,
   (i) providing a reaction system for detecting the level of expression of a gene selected from the group consisting of of zif 268, insulin-like growth factor, glutamate receptor 1 (GluR1), glutamate receptor 2 (GluR2), c/EBPβ and VGF;
   (ii) contacting said system with a test compound; and
   (iii) determining if the test compound alters the level of expression of the gene.

5. The method of claim 3 or 4, wherein the reaction system is a cell-free system.

6. The method of claim 5, wherein the cell-free system is purified protein preparation.

7. The method of claim 5, wherein the cell-free system is a cell-lysate.

8. The method of claim 3 or 4, wherein the reaction system is a whole cell system.

9. The method of claim 3 or 4, wherein the test agent is a organic molecule having a molecular weight less than 2500 amu.

10. The method of claim 3 or 4, which is repeated for a plurality of different test agents.

11. A pharmaceutical preparation comprising, one or more compounds identified in the assay of claim 3 or 4, formulated in a pharmaceutically acceptable excipient.

12. The pharmaceutical preparation of claim 11, further including one or more of a neuronal growth factor, a neuronal survival factor, and a neuronal tropic factor.

13. The pharmaceutical preparation of claim 11, further including an agent that activates CREB-dependent transcription in an amount sufficient to produce a memory enhancing effect.

14. A method for enhancing memory consolidation in an animal, or otherwise enhancing the functional performance of CNS neurons, comprising administering to an animal a pharmaceutical preparation of claim 11.

15. A method for augmenting learning and memory, or otherwise enhancing the functional performance of CNS neurons, comprising administering to an animal a pharmaceutical preparation of claim 11.

16. The method of claim 14 or 15, further comprising administering, conjointly with the pharmaceutical preparation, one or more of a neuronal growth factor, a neuronal survival factor, and a neuronal tropic factor.

17. The method of claim 14 or 15, further comprising administering, conjointly with the pharmaceutical preparation, an agent that activates CREB-dependent transcription in an amount sufficient to produce a memory enhancing effect.

18. The method of claim 17, wherein the CREB activating agent is a cAMP elevating agent.

19. The method of claim 18, wherein at least one cAMP agonist activates adenylate cyclase.

20. The method of claim 17, wherein the CREB activating agent is a cAMP analog.

21. The method of claim 17, wherein the CREB activating agent is a cAMP phosphodiesterase inhibitor.

22. A method for assessing a patient for learning and/or memory functional performance including a step of detecting the expression of a gene selected from the group consisting of of zif 268, insulin-like growth factor, glutamate receptor 1 (GluR1), glutamate receptor 2 (GluR2), c/EBPβ and VGF, or the level of activity of a gene product thereof, in the patient's hippocampus.

23. A method for assessing a patient for learning and/or memory functional performance including a step of detecting the expression of, or a mutation in, one or more genes selected from the group consisting of zif 268, insulin-like growth factor, glutamate receptor 1 (GluR1), glutamate receptor 2 (GluR2), c/EBPβ and VGF, or the level of activity of the gene products thereof, (optionally) in the patient's hippocampus.

24. A method of conducting a drug discovery business comprising:

(i) identifying, by the assay of claim 3 or 4, a test compound which the level of expression of the gene or the activity of the gene product;

(ii) conducting therapeutic profiling of agents identified in step (i), or further analogs thereof, for efficacy and toxicity in animals; and

(iii) formulating a pharmaceutical preparation including one or more agents identified in step (ii) as having an acceptable therapeutic profile.

25. The method of claim 24, including an additional step of establishing a distribution system for distributing the pharmaceutical preparation for sale, and may optionally include establishing a sales group for marketing the pharmaceutical preparation.

26. A method of conducting a target discovery business comprising:

(i) identifying, by the assay of claim 3 or 4, a test compound which the level of expression of the gene or the activity of the gene product;

(ii) (optionally) conducting therapeutic profiling of agents identified in step (i), or further analogs thereof, for efficacy and toxicity in animals; and

(iii). licensing, to a third party, the rights for further drug development of said identified agents.

Figure 1

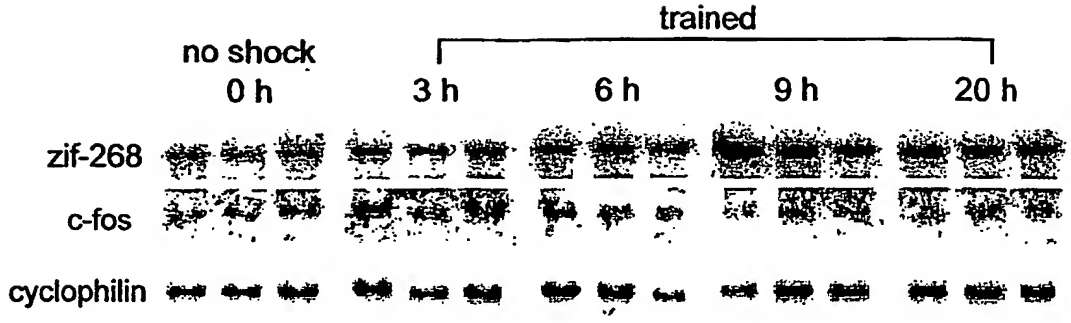
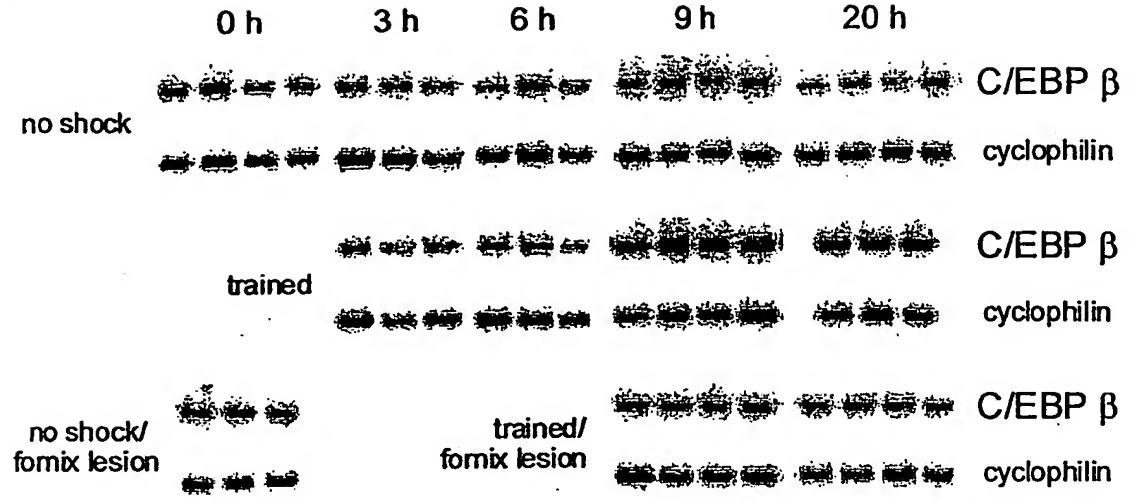


Figure 2

a


0 h
3 h
6 h
9 h
20 h
no shock
C/EBP β
cyclophilin
trained
C/EBP β
cyclophilin
no shock/
fornix lesion
trained/
fornix lesion
C/EBP β
cyclophilin

b

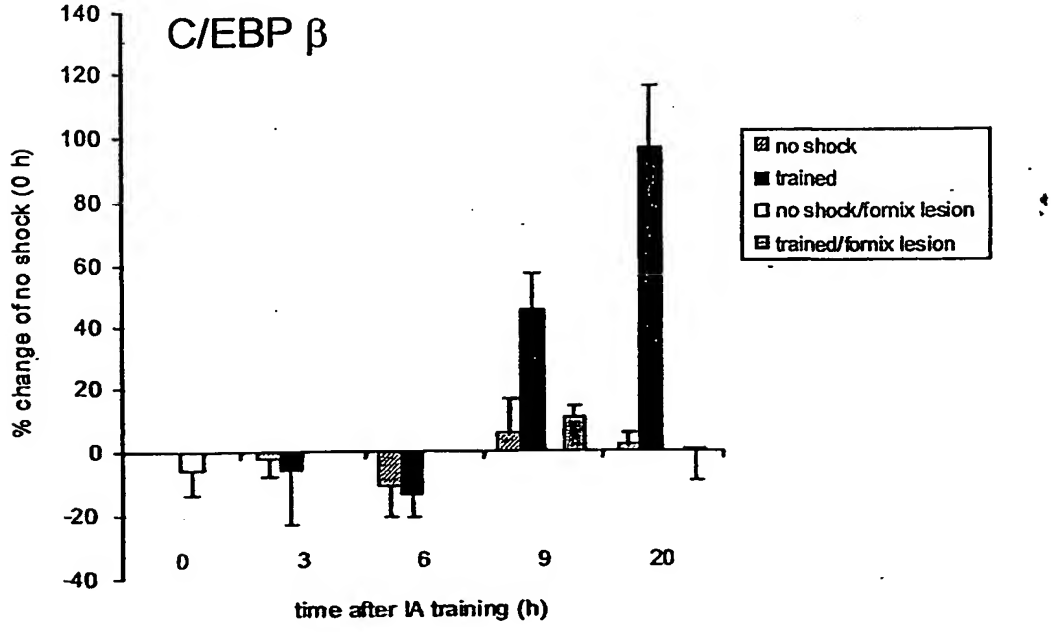

C/EBP β
% change of no shock (0 h)
140
120
100
80
60
40
20
0
-20
-40
0
3
6
9
20
time after IA training (h)
no shock
trained
no shock/fornix lesion
trained/fornix lesion

Figure 3

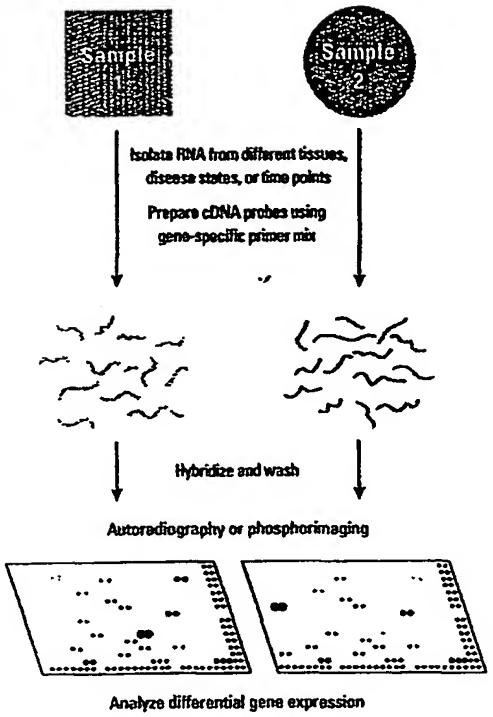

Sample 1
Sample 2
Isolate RNA from different tissues, disease states, or time points
Prepare cDNA probes using gene-specific primer mix
Hybridize and wash
Autoradiography or phosphorimaging
Analyze differential gene expression

Figure 4

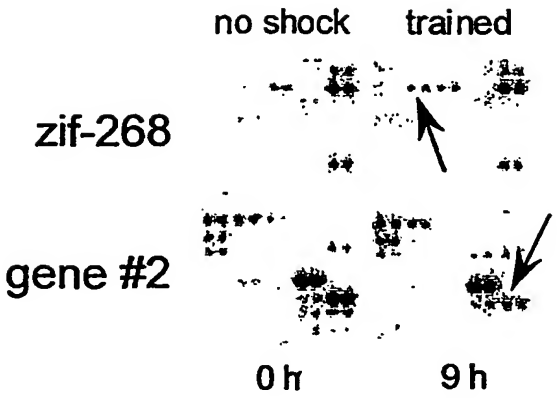
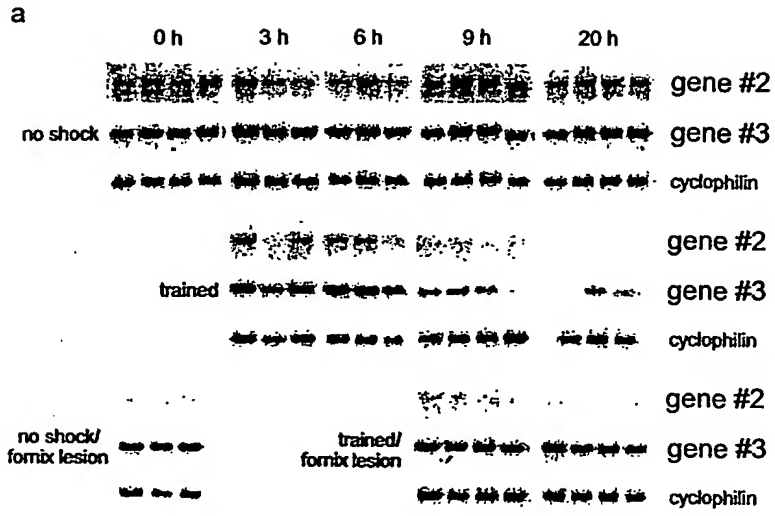


Figure 5



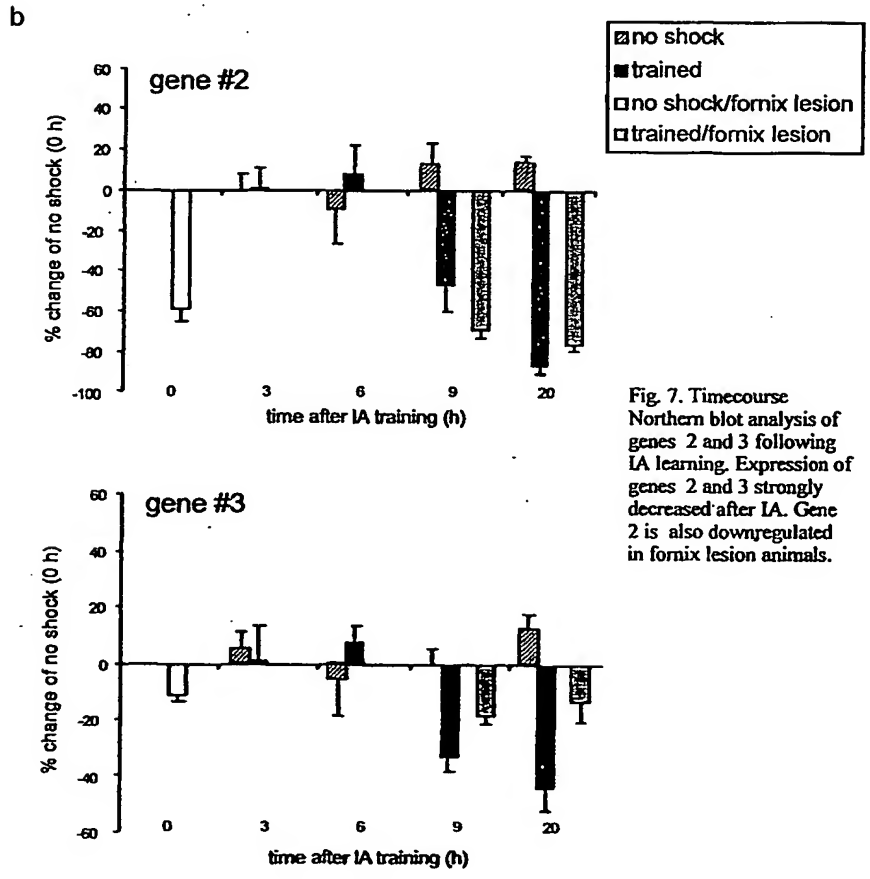
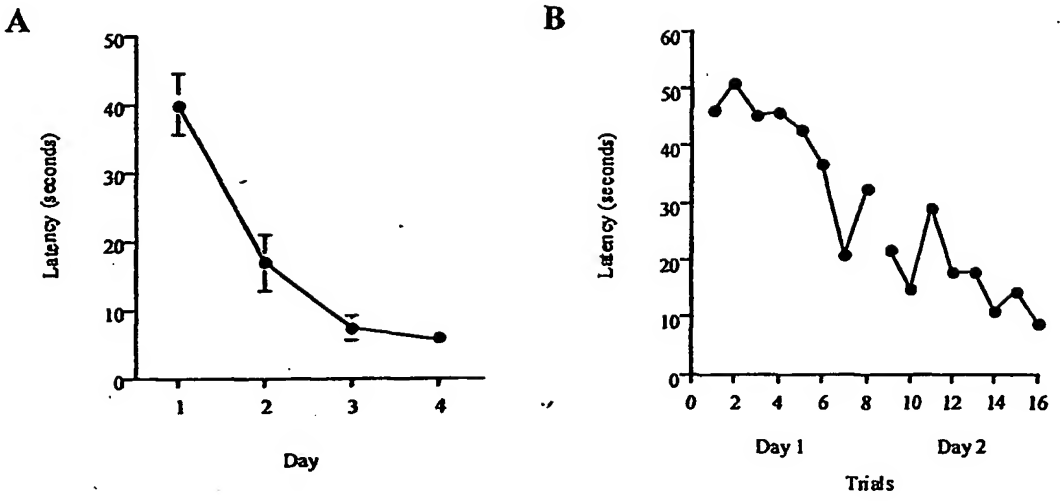


Fig. 7. Timecourse Northern blot analysis of genes 2 and 3 following IA learning. Expression of genes 2 and 3 strongly decreased after IA. Gene 2 is also downregulated in fornix lesion animals.

Figure 6


A
Latency (seconds)
50
40
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1
2
3
4
Day
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Latency (seconds)
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Trials

SEQUENCE LISTING

<110> BEAR, Mark
ALBERINI, Cristina

<120> METHODS AND COMPOSITIONS FOR REGULATING MEMORY CONSOLIDATION

<130> NIIN-pWO-008

<140> PCT/US01/000000
<141> 2000-04-02

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<151> 2000-03-31

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115
Met Gln His Ile
1

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163
Phe Ala Phe Phe Cys Thr Gly Phe Leu Gly Ala Val Val Gly Ala Asn
5 10 15 20

ttc ccc aac aat atc cag atc ggg gga tta ttt cca aac cag cag tca
211
Phe Pro Asn Asn Ile Gln Ile Gly Gly Leu Phe Pro Asn Gln Gln Ser
25 30 35

cag gaa cat gct gct ttt aga ttt gct ttg tcg caa ctc aca gag ccc
259
Gln Glu His Ala Ala Phe Arg Phe Ala Leu Ser Gln Leu Thr Glu Pro
40 45 50

ccg aag ctg ctc ccc cag att gat att gtg aac atc agc gac agc ttt
307
Pro Lys Leu Leu Pro Gln Ile Asp Ile Val Asn Ile Ser Asp Ser Phe
55 60 65

gag atg acc tat aga ttc tgt tcc cag ttc tcc aaa gga gtc tat gcc
355
Glu Met Thr Tyr Arg Phe Cys Ser Gln Phe Ser Lys Gly Val Tyr Ala
70 75 80

atc ttt ggg ttt tat gaa cgt agg act gtc aac atg ctg acc tcc ttt
403
Ile Phe Gly Phe Tyr Glu Arg Arg Thr Val Asn Met Leu Thr Ser Phe
85 90 95 100

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tgt ggg gcc ctc cac gtc tgc ttc att acg ccg agc ttt ccg gtt gat
451
Cys Gly Ala Leu His Val Cys Phe Ile Thr Pro Ser Phe Pro Val Asp
                105                 110                 115

aca tca aat cag ttt gtc ctt cag ctg cgc cct gaa ctg cag gat gcc
499
Thr Ser Asn Gln Phe Val Leu Gln Leu Arg Pro Glu Leu Gln Asp Ala
            120                 125                 130

ctc atc agc atc att gac cat tac aag tgg cag aaa ttt gtc tac att
547
Leu Ile Ser Ile Ile Asp His Tyr Lys Trp Gln Lys Phe Val Tyr Ile
        135                 140                 145

tat gat gcc gac cgg ggc tta tcc gtc ctg cag aaa gtc ctg gat aca
595
Tyr Asp Ala Asp Arg Gly Leu Ser Val Leu Gln Lys Val Leu Asp Thr
    150                 155                 160

gct gct gag aag aac tgg cag gtg aca gca gtc aac att ttg aca acc
643
Ala Ala Glu Lys Asn Trp Gln Val Thr Ala Val Asn Ile Leu Thr Thr
165                 170                 175                 180

aca gag gaa gga tac cgg atg ctc ttt cag gac ctg gag aag aaa aag
691
Thr Glu Glu Gly Tyr Arg Met Leu Phe Gln Asp Leu Glu Lys Lys Lys
                185                 190                 195

gag cgg ctg gtg gtg gtg gac tgt gaa tca gaa cgc ctc aat gct atc
739
Glu Arg Leu Val Val Val Asp Cys Glu Ser Glu Arg Leu Asn Ala Ile
            200                 205                 210

ttg ggc cag att ata aag cta gag aag aat ggc atc ggc tac cac tac
787
Leu Gly Gln Ile Ile Lys Leu Glu Lys Asn Gly Ile Gly Tyr His Tyr
        215                 220                 225

att ctt gca aat ctg ggc ttc atg gac att gac tta aac aaa ttc aag
835
Ile Leu Ala Asn Leu Gly Phe Met Asp Ile Asp Leu Asn Lys Phe Lys
    230                 235                 240

gag agt ggc gcc aat gtg aca ggt ttc cag ctg gtg aac tac aca gac
883
Glu Ser Gly Ala Asn Val Thr Gly Phe Gln Leu Val Asn Tyr Thr Asp
245                 250                 255                 260

act att ccg gcc aag atc atg cag cag tgg aag aat agt gat gct cga
931
Thr Ile Pro Ala Lys Ile Met Gln Gln Trp Lys Asn Ser Asp Ala Arg
                265                 270                 275

gac cac aca cgg gtg gac tgg aag aga ccc aag tac acc tct gcg ctc
979
Asp His Thr Arg Val Asp Trp Lys Arg Pro Lys Tyr Thr Ser Ala Leu
            280                 285                 290

acc tac gat ggg gtg aag gtg atg gct gag gct ttc cag agc ctg cgg
1027
Thr Tyr Asp Gly Val Lys Val Met Ala Glu Ala Phe Gln Ser Leu Arg
        295                 300                 305
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agg cag aga att gat ata tct cgc cgg ggg aat gct ggg gat tgt ctg
1075
Arg Gln Arg Ile Asp Ile Ser Arg Arg Gly Asn Ala Gly Asp Cys Leu
    310                 315                 320

gct aac cca gct gtt ccc tgg ggc caa ggg atc gac atc cag aga gct
1123
Ala Asn Pro Ala Val Pro Trp Gly Gln Gly Ile Asp Ile Gln Arg Ala
325                 330                 335                 340

ctg cag cag gtc gcg ttt gaa ggt tta aca gga aac gtg cag ttt aat
1171
Leu Gln Gln Val Ala Phe Glu Gly Leu Thr Gly Asn Val Gln Phe Asn
                345                 350                 355

gag aaa gga cgc cgg acc aac tac acg ctc cac gtg att gaa atg aaa
1219
Glu Lys Gly Arg Arg Thr Asn Tyr Thr Leu His Val Ile Glu Met Lys
            360                 365                 370

cat gac ggc atc cga aag att ggt tac tgg aat gaa gat gat aag ttt
1267
His Asp Gly Ile Arg Lys Ile Gly Tyr Trp Asn Glu Asp Asp Lys Phe
        375                 380                 385

gtc cct gca gcc acc gat gcc caa gct ggg ggg gat aat tca agt gtt
1315
Val Pro Ala Ala Thr Asp Ala Gln Ala Gly Gly Asp Asn Ser Ser Val
    390                 395                 400

cag aac aga aca tac atc gtc aca aca atc cta gaa gat cct tat gtg
1363
Gln Asn Arg Thr Tyr Ile Val Thr Thr Ile Leu Glu Asp Pro Tyr Val
405                 410                 415                 420

atg ctc aag aag aac gcc aat cag ttt gag ggc aat gac cgt tac gag
1411
Met Leu Lys Lys Asn Ala Asn Gln Phe Glu Gly Asn Asp Arg Tyr Glu
                425                 430                 435

ggc tac tgt gta gag ctg gcg gca gag att gcc aag cac gtg ggc tac
1459
Gly Tyr Cys Val Glu Leu Ala Ala Glu Ile Ala Lys His Val Gly Tyr
            440                 445                 450

tcc tac cgt ctg gag att gtc agt gat gga aaa tac gga gcc cga gac
1507
Ser Tyr Arg Leu Glu Ile Val Ser Asp Gly Lys Tyr Gly Ala Arg Asp
        455                 460                 465

cct gac acg aag gcc tgg aat ggc atg gtg gga gag ctg gtc tat gga
1555
Pro Asp Thr Lys Ala Trp Asn Gly Met Val Gly Glu Leu Val Tyr Gly
    470                 475                 480

aga gca gat gtg gct gtg gct ccc ctt act atc act ttg gtc cgg gaa
1603
Arg Ala Asp Val Ala Val Ala Pro Leu Thr Ile Thr Leu Val Arg Glu
485                 490                 495                 500

gaa gtt ata gat ttc tcc aaa cca ttt atg agt ttg ggg atc tcc atc
1651
Glu Val Ile Asp Phe Ser Lys Pro Phe Met Ser Leu Gly Ile Ser Ile
                505                 510                 515

atg att aaa aaa cca cag aaa tcc aag ccg ggt gtc ttc tcc ttc ctt
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1699
Met Ile Lys Lys Pro Gln Lys Ser Lys Pro Gly Val Phe Ser Phe Leu
520 525 530

gat cct ttg gct tat gag att tgg atg tgc att gtt ttt gcc tac att
1747
Asp Pro Leu Ala Tyr Glu Ile Trp Met Cys Ile Val Phe Ala Tyr Ile
535 540 545

gga gtg agt gtt gtc ctc ttc ctg gtc agc cgc ttc agt ccc tat gaa
1795
Gly Val Ser Val Val Leu Phe Leu Val Ser Arg Phe Ser Pro Tyr Glu
550 555 560

tgg cac agt gaa gag ttt gag gaa gga cgg gac cag aca acc agt gac
1843
Trp His Ser Glu Glu Phe Glu Glu Gly Arg Asp Gln Thr Thr Ser Asp
565 570 575 580

cag tcc aat gag ttt ggg ata ttc aac agt ttg tgg ttc tcc ctg gga
1891
Gln Ser Asn Glu Phe Gly Ile Phe Asn Ser Leu Trp Phe Ser Leu Gly
585 590 595

gcc ttc atg cag caa gga tgt gac att tct ccc agg tcc ctg tct ggt
1939
Ala Phe Met Gln Gln Gly Cys Asp Ile Ser Pro Arg Ser Leu Ser Gly
600 605 610

cgc atc gtc ggt ggc gtc tgg tgg ttc ttc acc tta atc atc atc tcc
1987
Arg Ile Val Gly Gly Val Trp Trp Phe Phe Thr Leu Ile Ile Ile Ser
615 620 625

tca tat aca gcc aat ctg gcc gcc ttc ctg acc gtg gag agg atg gtg
2035
Ser Tyr Thr Ala Asn Leu Ala Ala Phe Leu Thr Val Glu Arg Met Val
630 635 640

tct ccc att gag agt gca gag gac cta gcg aag cag aca gaa att gcc
2083
Ser Pro Ile Glu Ser Ala Glu Asp Leu Ala Lys Gln Thr Glu Ile Ala
645 650 655 660

tac ggg acg ctg gaa gca gga tct act aag gag ttc ttc agg agg tct
2131
Tyr Gly Thr Leu Glu Ala Gly Ser Thr Lys Glu Phe Phe Arg Arg Ser
665 670 675

aaa att gca gtg ttt gag aag atg tgg aca tac atg aag tca gca gag
2179
Lys Ile Ala Val Phe Glu Lys Met Trp Thr Tyr Met Lys Ser Ala Glu
680 685 690

cca tca gtt ttt gtg cgg aca aca gag gag ggg atg att cga gtg agg
2227
Pro Ser Val Phe Val Arg Thr Thr Glu Glu Gly Met Ile Arg Val Arg
695 700 705

aaa tcc aaa ggc aaa tat gcc tac ctc ctg gag tcc acc atg aat gag
2275
Lys Ser Lys Gly Lys Tyr Ala Tyr Leu Leu Glu Ser Thr Met Asn Glu
710 715 720

tac att gag cag cgg aaa ccc tgt gac acc atg aag gtg gga ggt aac
2323

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Tyr Ile Glu Gln Arg Lys Pro Cys Asp Thr Met Lys Val Gly Gly Asn
725                 730                 735                 740

ttg gat tcc aaa ggc tat ggc att gca aca ccc aag ggg tct gcc ctg
2371
Leu Asp Ser Lys Gly Tyr Gly Ile Ala Thr Pro Lys Gly Ser Ala Leu
                745                 750                 755

aga aat cca gta aac ctg gca gtg tta aaa ctg aac gag cag ggg ctt
2419
Arg Asn Pro Val Asn Leu Ala Val Leu Lys Leu Asn Glu Gln Gly Leu
            760                 765                 770

ttg gac aaa ttg aaa aac aaa tgg tgg tac gac aag ggc gag tgc ggc
2467
Leu Asp Lys Leu Lys Asn Lys Trp Trp Tyr Asp Lys Gly Glu Cys Gly
        775                 780                 785

agc ggg gga ggt gat tcc aag gac aag aca agc gct ctg agc ctc agc
2515
Ser Gly Gly Gly Asp Ser Lys Asp Lys Thr Ser Ala Leu Ser Leu Ser
    790                 795                 800

aat gtg gca ggc gtg ttc tac atc ctg atc gga gga ctt gga cta gcc
2563
Asn Val Ala Gly Val Phe Tyr Ile Leu Ile Gly Gly Leu Gly Leu Ala
805                 810                 815                 820

atg ctg gtt gcc tta atc gag ttc tgc tac aaa tcc cgt agt gaa tcc
2611
Met Leu Val Ala Leu Ile Glu Phe Cys Tyr Lys Ser Arg Ser Glu Ser
                825                 830                 835

aag cgg atg aag ggt ttt tgt ttg atc cca cag caa tcc atc aac gaa
2659
Lys Arg Met Lys Gly Phe Cys Leu Ile Pro Gln Gln Ser Ile Asn Glu
            840                 845                 850

gcc ata cgg aca tcg acc ctc ccc cgc aac agc ggg gca gga gcc agc
2707
Ala Ile Arg Thr Ser Thr Leu Pro Arg Asn Ser Gly Ala Gly Ala Ser
        855                 860                 865

agc ggc ggc agt gga gag aat ggt cgg gtg gtc agc cat gac ttc ccc
2755
Ser Gly Gly Ser Gly Glu Asn Gly Arg Val Val Ser His Asp Phe Pro
    870                 875                 880

aag tcc atg caa tcg att cct tgc atg agc cac agt tca ggg atg ccc
2803
Lys Ser Met Gln Ser Ile Pro Cys Met Ser His Ser Ser Gly Met Pro
885                 890                 895                 900

ttg gga gcc acg gga ttg taa ctggagcaga tggagacccc ttggggagca
2854
Leu Gly Ala Thr Gly Leu
                905

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2914

acgcaaccgg aattc
2929
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Asn Gln Gln Ser Gln Glu His Ala Ala Phe Arg Phe Ala Leu Ser Gln
35 40 45

Leu Thr Glu Pro Pro Lys Leu Leu Pro Gln Ile Asp Ile Val Asn Ile
50 55 60

Ser Asp Ser Phe Glu Met Thr Tyr Arg Phe Cys Ser Gln Phe Ser Lys
65 70 75 80

Gly Val Tyr Ala Ile Phe Gly Phe Tyr Glu Arg Arg Thr Val Asn Met
85 90 95

Leu Thr Ser Phe Cys Gly Ala Leu His Val Cys Phe Ile Thr Pro Ser
100 105 110

Phe Pro Val Asp Thr Ser Asn Gln Phe Val Leu Gln Leu Arg Pro Glu
115 120 125

Leu Gln Asp Ala Leu Ile Ser Ile Ile Asp His Tyr Lys Trp Gln Lys
130 135 140

Phe Val Tyr Ile Tyr Asp Ala Asp Arg Gly Leu Ser Val Leu Gln Lys
145 150 155 160

Val Leu Asp Thr Ala Ala Glu Lys Asn Trp Gln Val Thr Ala Val Asn
165 170 175

Ile Leu Thr Thr Thr Glu Glu Gly Tyr Arg Met Leu Phe Gln Asp Leu
180 185 190

Glu Lys Lys Lys Glu Arg Leu Val Val Val Asp Cys Glu Ser Glu Arg
195 200 205

Leu Asn Ala Ile Leu Gly Gln Ile Ile Lys Leu Glu Lys Asn Gly Ile
210 215 220

Gly Tyr His Tyr Ile Leu Ala Asn Leu Gly Phe Met Asp Ile Asp Leu
225 230 235 240

Asn Lys Phe Lys Glu Ser Gly Ala Asn Val Thr Gly Phe Gln Leu Val
245 250 255

Asn Tyr Thr Asp Thr Ile Pro Ala Lys Ile Met Gln Gln Trp Lys Asn
260 265 270

Ser Asp Ala Arg Asp His Thr Arg Val Asp Trp Lys Arg Pro Lys Tyr
275 280 285

Thr Ser Ala Leu Thr Tyr Asp Gly Val Lys Val Met Ala Glu Ala Phe
290 295 300

Gln Ser Leu Arg Arg Gln Arg Ile Asp Ile Ser Arg Arg Gly Asn Ala
305 310 315 320

Gly Asp Cys Leu Ala Asn Pro Ala Val Pro Trp Gly Gln Gly Ile Asp
325 330 335

Ile Gln Arg Ala Leu Gln Gln Val Ala Phe Glu Gly Leu Thr Gly Asn
340 345 350

Val Gln Phe Asn Glu Lys Gly Arg Arg Thr Asn Tyr Thr Leu His Val
355 360 365

Ile Glu Met Lys His Asp Gly Ile Arg Lys Ile Gly Tyr Trp Asn Glu
370 375 380

Asp Asp Lys Phe Val Pro Ala Ala Thr Asp Ala Gln Ala Gly Gly Asp
385 390 395 400

Asn Ser Ser Val Gln Asn Arg Thr Tyr Ile Val Thr Thr Ile Leu Glu
405 410 415

Asp Pro Tyr Val Met Leu Lys Lys Asn Ala Asn Gln Phe Glu Gly Asn
420 425 430

Asp Arg Tyr Glu Gly Tyr Cys Val Glu Leu Ala Ala Glu Ile Ala Lys
435 440 445

His Val Gly Tyr Ser Tyr Arg Leu Glu Ile Val Ser Asp Gly Lys Tyr
450 455 460

Gly Ala Arg Asp Pro Asp Thr Lys Ala Trp Asn Gly Met Val Gly Glu
465 470 475 480

Leu Val Tyr Gly Arg Ala Asp Val Ala Val Ala Pro Leu Thr Ile Thr
485 490 495

Leu Val Arg Glu Glu Val Ile Asp Phe Ser Lys Pro Phe Met Ser Leu
500 505 510

Gly Ile Ser Ile Met Ile Lys Lys Pro Gln Lys Ser Lys Pro Gly Val
515 520 525

Phe Ser Phe Leu Asp Pro Leu Ala Tyr Glu Ile Trp Met Cys Ile Val
530 535 540

Phe Ala Tyr Ile Gly Val Ser Val Val Leu Phe Leu Val Ser Arg Phe
545 550 555 560

Ser Pro Tyr Glu Trp His Ser Glu Glu Phe Glu Glu Gly Arg Asp Gln
565 570 575

Thr Thr Ser Asp Gln Ser Asn Glu Phe Gly Ile Phe Asn Ser Leu Trp
580 585 590

Phe Ser Leu Gly Ala Phe Met Gln Gln Gly Cys Asp Ile Ser Pro Arg
595 600 605

Ser Leu Ser Gly Arg Ile Val Gly Gly Val Trp Trp Phe Phe Thr Leu
610 615 620

Ile Ile Ile Ser Ser Tyr Thr Ala Asn Leu Ala Ala Phe Leu Thr Val
625 630 635 640

Glu Arg Met Val Ser Pro Ile Glu Ser Ala Glu Asp Leu Ala Lys Gln
645 650 655

Thr Glu Ile Ala Tyr Gly Thr Leu Glu Ala Gly Ser Thr Lys Glu Phe
660 665 670

Phe Arg Arg Ser Lys Ile Ala Val Phe Glu Lys Met Trp Thr Tyr Met
675 680 685

Lys Ser Ala Glu Pro Ser Val Phe Val Arg Thr Thr Glu Glu Gly Met
690 695 700

Ile Arg Val Arg Lys Ser Lys Gly Lys Tyr Ala Tyr Leu Leu Glu Ser
705 710 715 720

Thr Met Asn Glu Tyr Ile Glu Gln Arg Lys Pro Cys Asp Thr Met Lys
725 730 735

Val Gly Gly Asn Leu Asp Ser Lys Gly Tyr Gly Ile Ala Thr Pro Lys
740 745 750

Gly Ser Ala Leu Arg Asn Pro Val Asn Leu Ala Val Leu Lys Leu Asn
755 760 765

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Glu Gln Gly Leu Leu Asp Lys Leu Lys Asn Lys Trp Trp Tyr Asp Lys
    770                 775                 780

Gly Glu Cys Gly Ser Gly Gly Gly Asp Ser Lys Asp Lys Thr Ser Ala
785                 790                 795                 800

Leu Ser Leu Ser Asn Val Ala Gly Val Phe Tyr Ile Leu Ile Gly Gly
                805                 810                 815

Leu Gly Leu Ala Met Leu Val Ala Leu Ile Glu Phe Cys Tyr Lys Ser
            820                 825                 830

Arg Ser Glu Ser Lys Arg Met Lys Gly Phe Cys Leu Ile Pro Gln Gln
        835                 840                 845

Ser Ile Asn Glu Ala Ile Arg Thr Ser Thr Leu Pro Arg Asn Ser Gly
    850                 855                 860

Ala Gly Ala Ser Ser Gly Gly Ser Gly Glu Asn Gly Arg Val Val Ser
865                 870                 875                 880

His Asp Phe Pro Lys Ser Met Gln Ser Ile Pro Cys Met Ser His Ser
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Ser Gly Met Pro Leu Gly Ala Thr Gly Leu
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   1               5                   10                  15

gct gtg gct gag ggc cca gcc aag aag gtg ctg acc ctg gag gga gac
95
Ala Val Ala Glu Gly Pro Ala Lys Lys Val Leu Thr Leu Glu Gly Asp
                20                  25                  30

ttg gtg ctg ggt ggg ctg ttc cca gtg cac cag aag ggc ggc cca gca
143
Leu Val Leu Gly Gly Leu Phe Pro Val His Gln Lys Gly Gly Pro Ala
            35                  40                  45

gag gac tgt ggt cct gtc aat gag cac cgt ggc atc cag cgc ctg gag
191
Glu Asp Cys Gly Pro Val Asn Glu His Arg Gly Ile Gln Arg Leu Glu
        50                  55                  60
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gcc atg ctt ttt gca ctg gac cgc atc aac cgt gac ccg cac ctg ctg
239
Ala Met Leu Phe Ala Leu Asp Arg Ile Asn Arg Asp Pro His Leu Leu
 65                  70                  75

cct ggc gtg cgc ctg ggt gca cac atc ctc gac agt tgc tcc aag gac
287
Pro Gly Val Arg Leu Gly Ala His Ile Leu Asp Ser Cys Ser Lys Asp
80                  85                  90                  95

aca cat gcg ctg gag cag gca ctg gac ttt gtg cgt gcc tca ctc agc
335
Thr His Ala Leu Glu Gln Ala Leu Asp Phe Val Arg Ala Ser Leu Ser
                100                 105                 110

cgt ggt gct gat gga tca cgc cac atc tgc ccc gac ggc tct tat gcg
383
Arg Gly Ala Asp Gly Ser Arg His Ile Cys Pro Asp Gly Ser Tyr Ala
            115                 120                 125

acc cat ggt gat gct ccc act gcc atc act ggt gtt att ggc ggt tcc
431
Thr His Gly Asp Ala Pro Thr Ala Ile Thr Gly Val Ile Gly Gly Ser
        130                 135                 140

tac agt gat gtc tcc atc cag gtg gcc aac ctc ttg agg cta ttt cag
479
Tyr Ser Asp Val Ser Ile Gln Val Ala Asn Leu Leu Arg Leu Phe Gln
145                 150                 155

atc cca cag att agc tac gcc tct acc agt gcc aag ctg agt gac aag
527
Ile Pro Gln Ile Ser Tyr Ala Ser Thr Ser Ala Lys Leu Ser Asp Lys
160                 165                 170                 175

tcc cgc tat gac tac ttt gcc cgc aca gtg cct cct gac ttc ttc caa
575
Ser Arg Tyr Asp Tyr Phe Ala Arg Thr Val Pro Pro Asp Phe Phe Gln
                180                 185                 190

gcc aag gcc atg gct gag att ctc cgc ttc ttc aac tgg acc tat gtg
623
Ala Lys Ala Met Ala Glu Ile Leu Arg Phe Phe Asn Trp Thr Tyr Val
            195                 200                 205

tcc act gag gcc tct gag ggc gac tat ggc gag aca ggc att gag gcc
671
Ser Thr Glu Ala Ser Glu Gly Asp Tyr Gly Glu Thr Gly Ile Glu Ala
        210                 215                 220

ttt gag cta gag gct cgt gcc cgc aac atc tgt gtg gcc acc tcg gag
719
Phe Glu Leu Glu Ala Arg Ala Arg Asn Ile Cys Val Ala Thr Ser Glu
225                 230                 235

aaa gtg ggc cgt gcc atg agc cgc gcg gcc ttt gag ggt gtg gtg cga
767
Lys Val Gly Arg Ala Met Ser Arg Ala Ala Phe Glu Gly Val Val Arg
240                 245                 250                 255

gcc ctg ctg cag aag ccc agt gcc cgc gtg gct gtc ctg ttc acc cgt
815
Ala Leu Leu Gln Lys Pro Ser Ala Arg Val Ala Val Leu Phe Thr Arg
                260                 265                 270

tct gag gat gcc cgg gag ctg ctt gct gcc agc cag cgc ctc aat gcc
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```
863
Ser Glu Asp Ala Arg Glu Leu Leu Ala Ala Ser Gln Arg Leu Asn Ala
          275                 280                 285

agc ttc acc tgg gtg gcc agt gat ggt tgg ggg gcc ctg gag agt gtg
911
Ser Phe Thr Trp Val Ala Ser Asp Gly Trp Gly Ala Leu Glu Ser Val
        290                 295                 300

gtg gca ggc agt gag ggg gct gct gag ggt gct atc acc atc gag ctg
959
Val Ala Gly Ser Glu Gly Ala Ala Glu Gly Ala Ile Thr Ile Glu Leu
    305                 310                 315

gcc tcc tac ccc atc agt gac ttt gcc tcc tac ttc cag agc ctg gac
1007
Ala Ser Tyr Pro Ile Ser Asp Phe Ala Ser Tyr Phe Gln Ser Leu Asp
320                 325                 330                 335

cct tgg aac aac agc cgg aac ccc tgg ttc cgt gaa ttc tgg gag cag
1055
Pro Trp Asn Asn Ser Arg Asn Pro Trp Phe Arg Glu Phe Trp Glu Gln
                340                 345                 350

agg ttc cgc tgc agc ttc cgg cag cga gac tgc gca gcc cac tct ctc
1103
Arg Phe Arg Cys Ser Phe Arg Gln Arg Asp Cys Ala Ala His Ser Leu
            355                 360                 365

cgg gct gtg ccc ttt gaa cag gag tcc aag atc atg ttt gtg gtc aat
1151
Arg Ala Val Pro Phe Glu Gln Glu Ser Lys Ile Met Phe Val Val Asn
        370                 375                 380

gca gtg tac gcc atg gcc cat gcg ctc cac aac atg cac cgt gcc ctc
1199
Ala Val Tyr Ala Met Ala His Ala Leu His Asn Met His Arg Ala Leu
    385                 390                 395

tgc ccc aac acc acc cgg ctc tgt gac gcg atg cgg cca gtt aac ggg
1247
Cys Pro Asn Thr Thr Arg Leu Cys Asp Ala Met Arg Pro Val Asn Gly
400                 405                 410                 415

cgc cgc ctc tac aag gac ttt gtg ctc aac gtc aag ttt gat gcc ccc
1295
Arg Arg Leu Tyr Lys Asp Phe Val Leu Asn Val Lys Phe Asp Ala Pro
                420                 425                 430

ttt cgc cca gct gac acc cac aat gag gtc cgc ttt gac cgc ttt ggt
1343
Phe Arg Pro Ala Asp Thr His Asn Glu Val Arg Phe Asp Arg Phe Gly
            435                 440                 445

gat ggt att ggc cgc tac aac atc ttc acc tat ctg cgt gca ggc agt
1391
Asp Gly Ile Gly Arg Tyr Asn Ile Phe Thr Tyr Leu Arg Ala Gly Ser
        450                 455                 460

ggg cgc tat cgc tac cag aag gtg ggc tac tgg gca gaa ggc ttg act
1439
Gly Arg Tyr Arg Tyr Gln Lys Val Gly Tyr Trp Ala Glu Gly Leu Thr
    465                 470                 475

ctg gac acc agc ctc atc cca tgg gcc tca ccg tca gcc ggc ccc ctg
1487
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Leu Asp Thr Ser Leu Ile Pro Trp Ala Ser Pro Ser Ala Gly Pro Leu
480                 485                 490                 495

gcc gcc tct cgc tgc agt gag ccc tgc ctc cag aat gag gtg aag agt
1535
Ala Ala Ser Arg Cys Ser Glu Pro Cys Leu Gln Asn Glu Val Lys Ser
                500                 505                 510

gtg cag ccg ggc gaa gtc tgc tgc tgg ctc tgc att ccg tgc cag ccc
1583
Val Gln Pro Gly Glu Val Cys Cys Trp Leu Cys Ile Pro Cys Gln Pro
            515                 520                 525

tat gag tac cga ttg gac gaa ttc act tgc gct gat tgt ggc ctg ggc
1631
Tyr Glu Tyr Arg Leu Asp Glu Phe Thr Cys Ala Asp Cys Gly Leu Gly
        530                 535                 540

tac tgg ccc aat gcc agc ctg act ggc tgc ttc gaa ctg ccc cag gag
1679
Tyr Trp Pro Asn Ala Ser Leu Thr Gly Cys Phe Glu Leu Pro Gln Glu
    545                 550                 555

tac atc cgc tgg ggc gat gcc tgg gct gtg gga cct gtc acc atc gcc
1727
Tyr Ile Arg Trp Gly Asp Ala Trp Ala Val Gly Pro Val Thr Ile Ala
560                 565                 570                 575

tgc ctc ggt gcc ctg gcc acc ctg ttt gtg ctg ggt gtc ttt gtg cgg
1775
Cys Leu Gly Ala Leu Ala Thr Leu Phe Val Leu Gly Val Phe Val Arg
                580                 585                 590

cac aat gcc aca cca gtg gtc aag gcc tca ggt cgg gag ctc tgc tac
1823
His Asn Ala Thr Pro Val Val Lys Ala Ser Gly Arg Glu Leu Cys Tyr
            595                 600                 605

atc ctg ctg ggt ggt gtc ttc ctc tgc tac tgc atg acc ttc atc ttc
1871
Ile Leu Leu Gly Gly Val Phe Leu Cys Tyr Cys Met Thr Phe Ile Phe
        610                 615                 620

att gcc aag cca tcc acg gca gtg tgt acc tta cgg cgt ctt ggt ttg
1919
Ile Ala Lys Pro Ser Thr Ala Val Cys Thr Leu Arg Arg Leu Gly Leu
    625                 630                 635

ggc act gcc ttc tct gtc tgc tac tca gcc ctg ctc acc aag acc aac
1967
Gly Thr Ala Phe Ser Val Cys Tyr Ser Ala Leu Leu Thr Lys Thr Asn
640                 645                 650                 655

cgc att gca cgc atc ttc ggt ggg gcc cgg gag ggt gcc cag cgg cca
2015
Arg Ile Ala Arg Ile Phe Gly Gly Ala Arg Glu Gly Ala Gln Arg Pro
                660                 665                 670

cgc ttc atc agt cct gcc tca cag gtg gcc atc tgc ctg gca ctt atc
2063
Arg Phe Ile Ser Pro Ala Ser Gln Val Ala Ile Cys Leu Ala Leu Ile
            675                 680                 685

tcg ggc cag ctg ctc atc gtg gtc gcc tgg ctg gtg gtg gag gca ccg
2111
Ser Gly Gln Leu Leu Ile Val Val Ala Trp Leu Val Val Glu Ala Pro
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690 695 700

ggc aca ggc aag gag aca gcc ccc gaa cgg cgg gag gtg gtg aca ctg
2159
Gly Thr Gly Lys Glu Thr Ala Pro Glu Arg Arg Glu Val Val Thr Leu
705 710 715

cgc tgc aac cac cgc gat gca agt atg ttg ggc tcg ctg gcc tac aat
2207
Arg Cys Asn His Arg Asp Ala Ser Met Leu Gly Ser Leu Ala Tyr Asn
720 725 730 735

gtg ctc ctc atc gcg ctc tgc acg ctt tat gcc ttc aat act cgc aag
2255
Val Leu Leu Ile Ala Leu Cys Thr Leu Tyr Ala Phe Asn Thr Arg Lys
740 745 750

tgc ccc gaa aac ttc aac gag gcc aag ttc att ggc ttc acc atg tac
2303
Cys Pro Glu Asn Phe Asn Glu Ala Lys Phe Ile Gly Phe Thr Met Tyr
755 760 765

acc acc tgc atc atc tgg ctg gca ttg ttg ccc atc ttc tat gtc acc
2351
Thr Thr Cys Ile Ile Trp Leu Ala Leu Leu Pro Ile Phe Tyr Val Thr
770 775 780

tcc agt gac tac cgg gta cag acc acc acc atg tgc gtg tca gtc agc
2399
Ser Ser Asp Tyr Arg Val Gln Thr Thr Thr Met Cys Val Ser Val Ser
785 790 795

ctc agc ggc tcc gtg gtg ctt ggc tgc ctc ttt gcg ccc aag ctg cac
2447
Leu Ser Gly Ser Val Val Leu Gly Cys Leu Phe Ala Pro Lys Leu His
800 805 810 815

atc atc ctc ttc cag ccg cag aag aac gtg gtt agc cac cgg gca ccc
2495
Ile Ile Leu Phe Gln Pro Gln Lys Asn Val Val Ser His Arg Ala Pro
820 825 830

acc agc cgc ttt ggc agt gct gct gcc agg gcc agc tcc agc ctt ggc
2543
Thr Ser Arg Phe Gly Ser Ala Ala Ala Arg Ala Ser Ser Ser Leu Gly
835 840 845

caa ggg tct ggc tcc cag ttt gtc ccc act gtt tgc aat ggc cgt gag
2591
Gln Gly Ser Gly Ser Gln Phe Val Pro Thr Val Cys Asn Gly Arg Glu
850 855 860

gtg gtg gac tcg aca acg tca tcg ctt tga
2621
Val Val Asp Ser Thr Thr Ser Ser Leu
865 870

<210> 4
<211> 872
<212> PRT
<213> Homo sapiens

<400> 4

Met Gly Ser Leu Leu Ala Leu Leu Ala Leu Leu Pro Leu Trp Gly Ala

1 5 10 15

Val Ala Glu Gly Pro Ala Lys Lys Val Leu Thr Leu Glu Gly Asp Leu
20 25 30

Val Leu Gly Gly Leu Phe Pro Val His Gln Lys Gly Gly Pro Ala Glu
35 40 45

Asp Cys Gly Pro Val Asn Glu His Arg Gly Ile Gln Arg Leu Glu Ala
50 55 60

Met Leu Phe Ala Leu Asp Arg Ile Asn Arg Asp Pro His Leu Leu Pro
65 70 75 80

Gly Val Arg Leu Gly Ala His Ile Leu Asp Ser Cys Ser Lys Asp Thr
85 90 95

His Ala Leu Glu Gln Ala Leu Asp Phe Val Arg Ala Ser Leu Ser Arg
100 105 110

Gly Ala Asp Gly Ser Arg His Ile Cys Pro Asp Gly Ser Tyr Ala Thr
115 120 125

His Gly Asp Ala Pro Thr Ala Ile Thr Gly Val Ile Gly Gly Ser Tyr
130 135 140

Ser Asp Val Ser Ile Gln Val Ala Asn Leu Leu Arg Leu Phe Gln Ile
145 150 155 160

Pro Gln Ile Ser Tyr Ala Ser Thr Ser Ala Lys Leu Ser Asp Lys Ser
165 170 175

Arg Tyr Asp Tyr Phe Ala Arg Thr Val Pro Pro Asp Phe Phe Gln Ala
180 185 190

Lys Ala Met Ala Glu Ile Leu Arg Phe Phe Asn Trp Thr Tyr Val Ser
195 200 205

Thr Glu Ala Ser Glu Gly Asp Tyr Gly Glu Thr Gly Ile Glu Ala Phe
210 215 220

Glu Leu Glu Ala Arg Ala Arg Asn Ile Cys Val Ala Thr Ser Glu Lys
225 230 235 240

Val Gly Arg Ala Met Ser Arg Ala Ala Phe Glu Gly Val Val Arg Ala
245 250 255

Leu Leu Gln Lys Pro Ser Ala Arg Val Ala Val Leu Phe Thr Arg Ser
260 265 270

Glu Asp Ala Arg Glu Leu Leu Ala Ala Ser Gln Arg Leu Asn Ala Ser
275 280 285

Phe Thr Trp Val Ala Ser Asp Gly Trp Gly Ala Leu Glu Ser Val Val
290 295 300

Ala Gly Ser Glu Gly Ala Ala Glu Gly Ala Ile Thr Ile Glu Leu Ala
305 310 315 320

Ser Tyr Pro Ile Ser Asp Phe Ala Ser Tyr Phe Gln Ser Leu Asp Pro
325 330 335

Trp Asn Asn Ser Arg Asn Pro Trp Phe Arg Glu Phe Trp Glu Gln Arg
340 345 350

Phe Arg Cys Ser Phe Arg Gln Arg Asp Cys Ala Ala His Ser Leu Arg
355 360 365

Ala Val Pro Phe Glu Gln Glu Ser Lys Ile Met Phe Val Val Asn Ala
370 375 380

Val Tyr Ala Met Ala His Ala Leu His Asn Met His Arg Ala Leu Cys
385 390 395 400

Pro Asn Thr Thr Arg Leu Cys Asp Ala Met Arg Pro Val Asn Gly Arg
405 410 415

Arg Leu Tyr Lys Asp Phe Val Leu Asn Val Lys Phe Asp Ala Pro Phe
420 425 430

Arg Pro Ala Asp Thr His Asn Glu Val Arg Phe Asp Arg Phe Gly Asp
435 440 445

Gly Ile Gly Arg Tyr Asn Ile Phe Thr Tyr Leu Arg Ala Gly Ser Gly
450 455 460

Arg Tyr Arg Tyr Gln Lys Val Gly Tyr Trp Ala Glu Gly Leu Thr Leu
465 470 475 480

Asp Thr Ser Leu Ile Pro Trp Ala Ser Pro Ser Ala Gly Pro Leu Ala
485 490 495

Ala Ser Arg Cys Ser Glu Pro Cys Leu Gln Asn Glu Val Lys Ser Val
500 505 510

Gln Pro Gly Glu Val Cys Cys Trp Leu Cys Ile Pro Cys Gln Pro Tyr
515 520 525

Glu Tyr Arg Leu Asp Glu Phe Thr Cys Ala Asp Cys Gly Leu Gly Tyr

530 535 540

Trp Pro Asn Ala Ser Leu Thr Gly Cys Phe Glu Leu Pro Gln Glu Tyr
545 550 555 560

Ile Arg Trp Gly Asp Ala Trp Ala Val Gly Pro Val Thr Ile Ala Cys
565 570 575

Leu Gly Ala Leu Ala Thr Leu Phe Val Leu Gly Val Phe Val Arg His
580 585 590

Asn Ala Thr Pro Val Val Lys Ala Ser Gly Arg Glu Leu Cys Tyr Ile
595 600 605

Leu Leu Gly Gly Val Phe Leu Cys Tyr Cys Met Thr Phe Ile Phe Ile
610 615 620

Ala Lys Pro Ser Thr Ala Val Cys Thr Leu Arg Arg Leu Gly Leu Gly
625 630 635 640

Thr Ala Phe Ser Val Cys Tyr Ser Ala Leu Leu Thr Lys Thr Asn Arg
645 650 655

Ile Ala Arg Ile Phe Gly Gly Ala Arg Glu Gly Ala Gln Arg Pro Arg
660 665 670

Phe Ile Ser Pro Ala Ser Gln Val Ala Ile Cys Leu Ala Leu Ile Ser
675 680 685

Gly Gln Leu Leu Ile Val Val Ala Trp Leu Val Val Glu Ala Pro Gly
690 695 700

Thr Gly Lys Glu Thr Ala Pro Glu Arg Arg Glu Val Val Thr Leu Arg
705 710 715 720

Cys Asn His Arg Asp Ala Ser Met Leu Gly Ser Leu Ala Tyr Asn Val
725 730 735

Leu Leu Ile Ala Leu Cys Thr Leu Tyr Ala Phe Asn Thr Arg Lys Cys
740 745 750

Pro Glu Asn Phe Asn Glu Ala Lys Phe Ile Gly Phe Thr Met Tyr Thr
755 760 765

Thr Cys Ile Ile Trp Leu Ala Leu Leu Pro Ile Phe Tyr Val Thr Ser
770 775 780

Ser Asp Tyr Arg Val Gln Thr Thr Thr Met Cys Val Ser Val Ser Leu
785 790 795 800

Ser Gly Ser Val Val Leu Gly Cys Leu Phe Ala Pro Lys Leu His Ile
805 810 815

Ile Leu Phe Gln Pro Gln Lys Asn Val Val Ser His Arg Ala Pro Thr
820 825 830

Ser Arg Phe Gly Ser Ala Ala Ala Arg Ala Ser Ser Ser Leu Gly Gln
835 840 845

Gly Ser Gly Ser Gln Phe Val Pro Thr Val Cys Asn Gly Arg Glu Val
850 855 860

Val Asp Ser Thr Thr Ser Ser Leu
865 870

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<213> Homo sapiens

<220>
<221> CDS
<222> (271)..(1902)

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120

gcgagtcggg gtcgccgcct gcacgcttct cagtgttccc cgcgccccgc atgtaacccg
180

gccaggcccc cgcaacggtg tcccctgcag ctccagcccc gggctgcacc cccccgcccc
240

gacaccagct ctccagcctg ctcgtccagg atg gcc gcg gcc aag gcc gag atg
294
Met Ala Ala Ala Lys Ala Glu Met
1 5

cag ctg atg tcc ccg ctg cag atc tct gac ccg ttc gga tcc ttt cct
342
Gln Leu Met Ser Pro Leu Gln Ile Ser Asp Pro Phe Gly Ser Phe Pro
10 15 20

cac tcg ccc acc atg gac aac tac cct aag ctg gag gag atg atg ctg
390
His Ser Pro Thr Met Asp Asn Tyr Pro Lys Leu Glu Glu Met Met Leu
25 30 35 40

ctg agc aac ggg gct ccc cag ttc ctc ggc gcc gcc ggg gcc cca gag
438
Leu Ser Asn Gly Ala Pro Gln Phe Leu Gly Ala Ala Gly Ala Pro Glu
45 50 55

ggc agc ggc agc aac agc agc agc agc agc ggg ggc ggt gga ggc
486
Gly Ser Gly Ser Asn Ser Ser Ser Ser Ser Ser Gly Gly Gly Gly Gly

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            60                  65                  70

ggc ggg ggc ggc agc aac agc agc agc agc agc acc ttc aac cct
534
Gly Gly Gly Gly Ser Asn Ser Ser Ser Ser Ser Thr Phe Asn Pro
      75                  80                  85

cag gcg gac acg ggc gag cag ccc tac gag cac ctg acc gca gag tct
582
Gln Ala Asp Thr Gly Glu Gln Pro Tyr Glu His Leu Thr Ala Glu Ser
    90                  95                 100

ttt cct gac atc tct ctg aac aac gag aag gtg ctg gtg gag acc agt
630
Phe Pro Asp Ile Ser Leu Asn Asn Glu Lys Val Leu Val Glu Thr Ser
105                 110                 115                 120

tac ccc agc caa acc act cga ctg ccc ccc atc acc tat act ggc cgc
678
Tyr Pro Ser Gln Thr Thr Arg Leu Pro Pro Ile Thr Tyr Thr Gly Arg
                125                 130                 135

ttt tcc ctg gag cct gca ccc aac agt ggc aac acc ttg tgg ccc gag
726
Phe Ser Leu Glu Pro Ala Pro Asn Ser Gly Asn Thr Leu Trp Pro Glu
            140                 145                 150

ccc ctc ttc agc ttg gtc agt ggc cta gtg agc atg acc aac cca ccg
774
Pro Leu Phe Ser Leu Val Ser Gly Leu Val Ser Met Thr Asn Pro Pro
        155                 160                 165

gcc tcc tcg tcc tca gca cca tct cca gcg gcc tcc tcc gcc tcc gcc
822
Ala Ser Ser Ser Ser Ala Pro Ser Pro Ala Ala Ser Ser Ala Ser Ala
    170                 175                 180

tcc cag agc cca ccc ctg agc tgc gca gtg cca tcc aac gac agc agt
870
Ser Gln Ser Pro Pro Leu Ser Cys Ala Val Pro Ser Asn Asp Ser Ser
185                 190                 195                 200

ccc att tac tca gcg gca ccc acc ttc ccc acg ccg aac act gac att
918
Pro Ile Tyr Ser Ala Ala Pro Thr Phe Pro Thr Pro Asn Thr Asp Ile
                205                 210                 215

ttc cct gag cca caa agc cag gcc ttc ccg ggc tcg gca ggg aca gcg
966
Phe Pro Glu Pro Gln Ser Gln Ala Phe Pro Gly Ser Ala Gly Thr Ala
            220                 225                 230

ctc cag tac ccg cct cct gcc tac cct gcc gcc aag ggt ggc ttc cag
1014
Leu Gln Tyr Pro Pro Pro Ala Tyr Pro Ala Ala Lys Gly Gly Phe Gln
        235                 240                 245

gtt ccc atg atc ccc gac tac ctg ttt cca cag cag cag ggg gat ctg
1062
Val Pro Met Ile Pro Asp Tyr Leu Phe Pro Gln Gln Gln Gly Asp Leu
    250                 255                 260

ggc ctg ggc acc cca gac cag aag ccc ttc cag ggc ctg gag agc cgc
1110
Gly Leu Gly Thr Pro Asp Gln Lys Pro Phe Gln Gly Leu Glu Ser Arg
265                 270                 275                 280
```

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acc cag cag cct tcg cta acc cct ctg tct act att aag gcc ttt gcc
1158
Thr Gln Gln Pro Ser Leu Thr Pro Leu Ser Thr Ile Lys Ala Phe Ala
                285                 290                 295

act cag tcg ggc tcc cag gac ctg aag gcc ctc aat acc agc tac cag
1206
Thr Gln Ser Gly Ser Gln Asp Leu Lys Ala Leu Asn Thr Ser Tyr Gln
            300                 305                 310

tcc cag ctc atc aaa ccc agc cgc atg cgc aag tat ccc aac cgg ccc
1254
Ser Gln Leu Ile Lys Pro Ser Arg Met Arg Lys Tyr Pro Asn Arg Pro
        315                 320                 325

agc aag acg ccc ccc cac gaa cgc cct tac gct tgc cca gtg gag tcc
1302
Ser Lys Thr Pro Pro His Glu Arg Pro Tyr Ala Cys Pro Val Glu Ser
    330                 335                 340

tgt gat cgc cgc ttc tcc cgc tcc gac gag ctc acc cgc cac atc cgc
1350
Cys Asp Arg Arg Phe Ser Arg Ser Asp Glu Leu Thr Arg His Ile Arg
345                 350                 355                 360

atc cac aca ggc cag aag ccc ttc cag tgc cgc atc tgc atg cgc aac
1398
Ile His Thr Gly Gln Lys Pro Phe Gln Cys Arg Ile Cys Met Arg Asn
                365                 370                 375

ttc agc cgc agc gac cac ctc acc acc cac atc cgc acc cac aca ggc
1446
Phe Ser Arg Ser Asp His Leu Thr Thr His Ile Arg Thr His Thr Gly
            380                 385                 390

gaa aag ccc ttc gcc tgc gac atc tgt gga aga aag ttt gcc agg agc
1494
Glu Lys Pro Phe Ala Cys Asp Ile Cys Gly Arg Lys Phe Ala Arg Ser
        395                 400                 405

gat gaa cgc aag agg cat acc aag atc cac ttg cgg cag aag gac aag
1542
Asp Glu Arg Lys Arg His Thr Lys Ile His Leu Arg Gln Lys Asp Lys
    410                 415                 420

aaa gca gac aaa agt gtt gtg gcc tct tcg gcc acc tcc tct ctc tct
1590
Lys Ala Asp Lys Ser Val Val Ala Ser Ser Ala Thr Ser Ser Leu Ser
425                 430                 435                 440

tcc tac ccg tcc ccg gtt gct acc tct tac ccg tcc ccg gtt act acc
1638
Ser Tyr Pro Ser Pro Val Ala Thr Ser Tyr Pro Ser Pro Val Thr Thr
                445                 450                 455

tct tat cca tcc ccg gcc acc acc tca tac cca tcc cct gtg ccc acc
1686
Ser Tyr Pro Ser Pro Ala Thr Thr Ser Tyr Pro Ser Pro Val Pro Thr
            460                 465                 470

tcc ttc tcc tct ccc ggc tcc tcg acc tac cca tcc cct gtg cac agt
1734
Ser Phe Ser Ser Pro Gly Ser Ser Thr Tyr Pro Ser Pro Val His Ser
        475                 480                 485
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ggc ttc ccc tcc ccg tcg gtg gcc acc acg tac tcc tct gtt ccc cct
1782
Gly Phe Pro Ser Pro Ser Val Ala Thr Thr Tyr Ser Ser Val Pro Pro
    490                 495                 500

gct ttc ccg gcc cag gtc agc agc ttc cct tcc tca gct gtc acc aac
1830
Ala Phe Pro Ala Gln Val Ser Ser Phe Pro Ser Ser Ala Val Thr Asn
505                 510                 515                 520

tcc ttc agc gcc tcc aca ggg ctt tcg gac atg aca gca acc ttt tct
1878
Ser Phe Ser Ala Ser Thr Gly Leu Ser Asp Met Thr Ala Thr Phe Ser
                525                 530                 535

ccc agg aca att gaa att tgc taa agggaaaggg gaaagaaagg gaaaagggag
1932
Pro Arg Thr Ile Glu Ile Cys
            540

aaaaagaaac acaagagact taaaggacag gaggaggaga tggccatagg agaggagggt
1992

tcctcttagg tcagatggag gttctcagag ccaagtcctc cctctctact ggagtggaag
2052

gtctattggc caacaatcct ttctgcccac ttcccccttcc ccaattacta ttccctttga
2112

cttcagctgc ctgaaacagc catgtccaag ttcttcacct ctatccaaag aacttgattt
2172

gcatggattt tggataaatc atttcagtat catctccatc atatgcctga ccccttgctc
2232

ccttcaatgc tagaaaatcg agttggcaaa atggggtttg ggcccctcag agccctgccc
2292

tgcacccttg tacagtgtct gtgccatgga tttcgttttt cttggggtac tcttgatgtg
2352

aagataattt gcatattcta ttgtattatt tggagttagg tcctcacttg ggggaaaaaa
2412

aaaaaaaaaa gccaagcaaa ccaatggtga tcctctattt tgtgatgatg ctgtgacaat
2472

aagtttgaac ctttttttttt gaaacagcag tcccagtatt ctcagagcat gtgtcagagt
2532

gttgttccgt taacctttttt gtaaatactg cttgaccgta ctctcacatg tggcaaaata
2592

tggtttggtt tttctttttt ttttttgaaa gtgtttttttc ttcgtccttt tggtttaaaa
2652

agtttcacgt cttggtgcct tttgtgtgat gccccttgct gatggcttga catgtgcaat
2712

tgtgagggac atgctcacct ctagccttaa ggggggcagg gagtgatgat ttgggggagg
2772

ctttgggagc aaaataagga agagggctga gctgagcttc ggttctccag aatgtaagaa
2832

aacaaaatct aaaacaaaat ctgaactctc aaaagtctat ttttttaact gaaaatgtaa
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2892

atttataaat atattcagga gttggaatgt tgtagttacc tactgagtag gcggcgattt
2952

ttgtatgtta tgaacatgca gttcattatt ttgtggttct attttacttt gtacttgtgt
3012

ttgcttaaac aaagtgactg tttggcttat aaacacattg aatgcgcttt attgcccatg
3072

ggatatgtgg tgtatatcct tccaaaaaat taaaacgaaa ataaagtagc tgcgattggg
3132

<210> 6
<211> 543
<212> PRT
<213> Homo sapiens

<400> 6

Met Ala Ala Ala Lys Ala Glu Met Gln Leu Met Ser Pro Leu Gln Ile
1 5 10 15

Ser Asp Pro Phe Gly Ser Phe Pro His Ser Pro Thr Met Asp Asn Tyr
20 25 30

Pro Lys Leu Glu Glu Met Met Leu Leu Ser Asn Gly Ala Pro Gln Phe
35 40 45

Leu Gly Ala Ala Gly Ala Pro Glu Gly Ser Gly Ser Asn Ser Ser Ser
50 55 60

Ser Ser Ser Gly Gly Gly Gly Gly Gly Gly Gly Gly Ser Asn Ser Ser
65 70 75 80

Ser Ser Ser Ser Thr Phe Asn Pro Gln Ala Asp Thr Gly Glu Gln Pro
85 90 95

Tyr Glu His Leu Thr Ala Glu Ser Phe Pro Asp Ile Ser Leu Asn Asn
100 105 110

Glu Lys Val Leu Val Glu Thr Ser Tyr Pro Ser Gln Thr Thr Arg Leu
115 120 125

Pro Pro Ile Thr Tyr Thr Gly Arg Phe Ser Leu Glu Pro Ala Pro Asn
130 135 140

Ser Gly Asn Thr Leu Trp Pro Glu Pro Leu Phe Ser Leu Val Ser Gly
145 150 155 160

Leu Val Ser Met Thr Asn Pro Pro Ala Ser Ser Ser Ser Ala Pro Ser
165 170 175

Pro Ala Ala Ser Ser Ala Ser Ala Ser Gln Ser Pro Pro Leu Ser Cys
180 185 190

Ala Val Pro Ser Asn Asp Ser Ser Pro Ile Tyr Ser Ala Ala Pro Thr
195 200 205

Phe Pro Thr Pro Asn Thr Asp Ile Phe Pro Glu Pro Gln Ser Gln Ala
210 215 220

Phe Pro Gly Ser Ala Gly Thr Ala Leu Gln Tyr Pro Pro Pro Ala Tyr
225 230 235 240

Pro Ala Ala Lys Gly Gly Phe Gln Val Pro Met Ile Pro Asp Tyr Leu
245 250 255

Phe Pro Gln Gln Gln Gly Asp Leu Gly Leu Gly Thr Pro Asp Gln Lys
260 265 270

Pro Phe Gln Gly Leu Glu Ser Arg Thr Gln Gln Pro Ser Leu Thr Pro
275 280 285

Leu Ser Thr Ile Lys Ala Phe Ala Thr Gln Ser Gly Ser Gln Asp Leu
290 295 300

Lys Ala Leu Asn Thr Ser Tyr Gln Ser Gln Leu Ile Lys Pro Ser Arg
305 310 315 320

Met Arg Lys Tyr Pro Asn Arg Pro Ser Lys Thr Pro Pro His Glu Arg
325 330 335

Pro Tyr Ala Cys Pro Val Glu Ser Cys Asp Arg Arg Phe Ser Arg Ser
340 345 350

Asp Glu Leu Thr Arg His Ile Arg Ile His Thr Gly Gln Lys Pro Phe
355 360 365

Gln Cys Arg Ile Cys Met Arg Asn Phe Ser Arg Ser Asp His Leu Thr
370 375 380

Thr His Ile Arg Thr His Thr Gly Glu Lys Pro Phe Ala Cys Asp Ile
385 390 395 400

Cys Gly Arg Lys Phe Ala Arg Ser Asp Glu Arg Lys Arg His Thr Lys
405 410 415

Ile His Leu Arg Gln Lys Asp Lys Lys Ala Asp Lys Ser Val Val Ala
420 425 430

Ser Ser Ala Thr Ser Ser Leu Ser Ser Tyr Pro Ser Pro Val Ala Thr
435 440 445

Ser Tyr Pro Ser Pro Val Thr Thr Ser Tyr Pro Ser Pro Ala Thr Thr
450 455 460

Ser Tyr Pro Ser Pro Val Pro Thr Ser Phe Ser Ser Pro Gly Ser Ser
465 470 475 480

Thr Tyr Pro Ser Pro Val His Ser Gly Phe Pro Ser Pro Ser Val Ala
485 490 495

Thr Thr Tyr Ser Ser Val Pro Pro Ala Phe Pro Ala Gln Val Ser Ser
500 505 510

Phe Pro Ser Ser Ala Val Thr Asn Ser Phe Ser Ala Ser Thr Gly Leu
515 520 525

Ser Asp Met Thr Ala Thr Phe Ser Pro Arg Thr Ile Glu Ile Cys
530 535 540

<210> 7
<211> 612
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (151)..(564)

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120

cctgctgtaa aagacctgga acaaacaaaa atg att aca cct aca gtg aag atg
174
Met Ile Thr Pro Thr Val Lys Met
1 5

cac acc atg tcc tcc tcg cat ctc ttc tac ctg gcg ctg tgc ctg ctc
222
His Thr Met Ser Ser Ser His Leu Phe Tyr Leu Ala Leu Cys Leu Leu
10 15 20

acc ttc acc agc tct gcc acg gct gga ccg gag acg ctc tgc ggg gct
270
Thr Phe Thr Ser Ser Ala Thr Ala Gly Pro Glu Thr Leu Cys Gly Ala
25 30 35 40

gag ctg gtg gat gct ctt cag ttc gtg tgt gga gac agg ggc ttt tat
318
Glu Leu Val Asp Ala Leu Gln Phe Val Cys Gly Asp Arg Gly Phe Tyr
45 50 55

ttc aac aag ccc aca ggg tat ggc tcc agc agt cgg agg gcg cct cag
366
Phe Asn Lys Pro Thr Gly Tyr Gly Ser Ser Ser Arg Arg Ala Pro Gln
60 65 70

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aca ggc atc gtg gat gag tgc tgc ttc cgg agc tgt gat cta agg agg
414
Thr Gly Ile Val Asp Glu Cys Cys Phe Arg Ser Cys Asp Leu Arg Arg
        75                  80                  85

ctg gag atg tat tgc gca ccc ctc aag cct gcc aag tca gct cgc tct
462
Leu Glu Met Tyr Cys Ala Pro Leu Lys Pro Ala Lys Ser Ala Arg Ser
    90                  95                  100

gtc cgt gcc cag cgc cac acc gac atg ccc aag acc cag aag gaa gta
510
Val Arg Ala Gln Arg His Thr Asp Met Pro Lys Thr Gln Lys Glu Val
105                 110                 115                 120

cat ttg aag aac gca agt aga ggg agt gca gga aac aag aac tac agg
558
His Leu Lys Asn Ala Ser Arg Gly Ser Ala Gly Asn Lys Asn Tyr Arg
                125                 130                 135

atg tag gaagaccctc ctgaggagtg aagagtgaca tgccaccgca ggatcctt
612
Met
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<210> 8
<211> 137
<212> PRT
<213> Homo sapiens

<400> 8

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Met Ile Thr Pro Thr Val Lys Met His Thr Met Ser Ser Ser His Leu
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Phe Tyr Leu Ala Leu Cys Leu Leu Thr Phe Thr Ser Ser Ala Thr Ala
            20                  25                  30

Gly Pro Glu Thr Leu Cys Gly Ala Glu Leu Val Asp Ala Leu Gln Phe
        35                  40                  45

Val Cys Gly Asp Arg Gly Phe Tyr Phe Asn Lys Pro Thr Gly Tyr Gly
    50                  55                  60

Ser Ser Ser Arg Arg Ala Pro Gln Thr Gly Ile Val Asp Glu Cys Cys
65                  70                  75                  80

Phe Arg Ser Cys Asp Leu Arg Arg Leu Glu Met Tyr Cys Ala Pro Leu
                85                  90                  95

Lys Pro Ala Lys Ser Ala Arg Ser Val Arg Ala Gln Arg His Thr Asp
            100                 105                 110

Met Pro Lys Thr Gln Lys Glu Val His Leu Lys Asn Ala Ser Arg Gly
        115                 120                 125
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Ser Ala Gly Asn Lys Asn Tyr Arg Met
130 135

<210> 9
<211> 1038
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (1)..(1038)

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48
Met Gln Arg Leu Val Ala Trp Asp Pro Ala Cys Leu Pro Leu Pro Pro
1 5 10 15

ccg ccg cct gcc ttt aaa tcc atg gaa gtg gcc aac ttc tac tac gag
96
Pro Pro Pro Ala Phe Lys Ser Met Glu Val Ala Asn Phe Tyr Tyr Glu
20 25 30

gcg gac tgc ttg gct gct gcg tac ggc ggc aag gcg gcc ccc gcg gcg
144
Ala Asp Cys Leu Ala Ala Ala Tyr Gly Gly Lys Ala Ala Pro Ala Ala
35 40 45

ccc ccc gcg gcc aga ccc ggg ccg cgc ccc.ccc gcc ggc gag ctg ggc
192
Pro Pro Ala Ala Arg Pro Gly Pro Arg Pro Pro Ala Gly Glu Leu Gly
50 55 60

agc atc ggc gac cac gag cgc gcc atc gac ttc agc ccg tac ctg gag
240
Ser Ile Gly Asp His Glu Arg Ala Ile Asp Phe Ser Pro Tyr Leu Glu
65 70 75 80

ccg ctg ggc gcg ccg cag gcc ccg gcg ccc gcc acg gcc acg gac acc
288
Pro Leu Gly Ala Pro Gln Ala Pro Ala Pro Ala Thr Ala Thr Asp Thr
85 90 95

ttc gag gcg gct ccg ccc gcg ccc gcc ccc gcg ccc gcc tcc tcc ggg
336
Phe Glu Ala Ala Pro Pro Ala Pro Ala Pro Ala Pro Ala Ser Ser Gly
100 105 110

cag cac cac gac ttc ctc tcc gac ctc ttc tcc gac gac tac ggg ggc
384
Gln His His Asp Phe Leu Ser Asp Leu Phe Ser Asp Asp Tyr Gly Gly
115 120 125

aag aac tgc aag aag ccg gcc gag tac ggc tac gtg agc ctg ggg cgc
432
Lys Asn Cys Lys Lys Pro Ala Glu Tyr Gly Tyr Val Ser Leu Gly Arg
130 135 140

ctg ggg gct gcc aag ggc gcg ctg cac ccc ggc tgc ttc gcg ccc ctg
480
Leu Gly Ala Ala Lys Gly Ala Leu His Pro Gly Cys Phe Ala Pro Leu
145 150 155 160

cac cca ccg ccc ccg ccg ccg ccg ccg ccc gcc gag ctc aag gcg gag
528

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His Pro Pro Pro Pro Pro Pro Pro Pro Ala Glu Leu Lys Ala Glu
                165                 170                 175

ccg ggc ttc gag ccc gcg gac tgc aag cgg aag gag gag gcc ggg gcg
576
Pro Gly Phe Glu Pro Ala Asp Cys Lys Arg Lys Glu Glu Ala Gly Ala
            180                 185                 190

ccg ggc ggc ggc gca ggc atg gcg gcg ggc ttc ccg tac gcg ctg cgc
624
Pro Gly Gly Gly Ala Gly Met Ala Ala Gly Phe Pro Tyr Ala Leu Arg
        195                 200                 205

gct tac ctc ggc tac cag gcg gtg ccg agc ggc agc agc ggg agc ctc
672
Ala Tyr Leu Gly Tyr Gln Ala Val Pro Ser Gly Ser Ser Gly Ser Leu
    210                 215                 220

tcc acg tcc tcc tcg tcc agc ccg ccc ggc acg ccg agc ccc gct gac
720
Ser Thr Ser Ser Ser Ser Ser Pro Pro Gly Thr Pro Ser Pro Ala Asp
225                 230                 235                 240

gcc aag gcc ccc ccg acc gcc tgc tac gcg ggg gcc ggg ccg gcg ccc
768
Ala Lys Ala Pro Pro Thr Ala Cys Tyr Ala Gly Ala Gly Pro Ala Pro
                245                 250                 255

tcg cag gtc aag agc aag gcc aag aag acc gtg gac aag cac agc gac
816
Ser Gln Val Lys Ser Lys Ala Lys Lys Thr Val Asp Lys His Ser Asp
            260                 265                 270

gag tac aag atc cgg cgc gag cgc aac aac atc gcc gtg cgc aag agc
864
Glu Tyr Lys Ile Arg Arg Glu Arg Asn Asn Ile Ala Val Arg Lys Ser
        275                 280                 285

cgc gac aag gcc aag atg cgc aac ctg gag acg cag cac aag gtc ctg
912
Arg Asp Lys Ala Lys Met Arg Asn Leu Glu Thr Gln His Lys Val Leu
    290                 295                 300

gag ctc acg gcc gag aac gag cgg ctg cag aag aag gtg gag cag ctg
960
Glu Leu Thr Ala Glu Asn Glu Arg Leu Gln Lys Lys Val Glu Gln Leu
305                 310                 315                 320

tcg cgc gag ctc agc acc ctg cgg aac ttg ttc aag cag ctg ccc gag
1008
Ser Arg Glu Leu Ser Thr Leu Arg Asn Leu Phe Lys Gln Leu Pro Glu
                325                 330                 335

ccc ctg ctc gcc tcc tcc ggc cac tgc tag
1038
Pro Leu Leu Ala Ser Ser Gly His Cys
            340                 345
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<210> 10
<211> 345
<212> PRT
<213> Homo sapiens

<400> 10

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Met Gln Arg Leu Val Ala Trp Asp Pro Ala Cys Leu Pro Leu Pro Pro
1               5                  10                  15

Pro Pro Pro Ala Phe Lys Ser Met Glu Val Ala Asn Phe Tyr Tyr Glu
            20                  25                  30

Ala Asp Cys Leu Ala Ala Ala Tyr Gly Gly Lys Ala Ala Pro Ala Ala
        35                  40                  45

Pro Pro Ala Ala Arg Pro Gly Pro Arg Pro Pro Ala Gly Glu Leu Gly
    50                  55                  60

Ser Ile Gly Asp His Glu Arg Ala Ile Asp Phe Ser Pro Tyr Leu Glu
65                  70                  75                  80

Pro Leu Gly Ala Pro Gln Ala Pro Ala Pro Ala Thr Ala Thr Asp Thr
                85                  90                  95

Phe Glu Ala Ala Pro Pro Ala Pro Ala Pro Ala Pro Ala Ser Ser Gly
            100                 105                 110

Gln His His Asp Phe Leu Ser Asp Leu Phe Ser Asp Asp Tyr Gly Gly
        115                 120                 125

Lys Asn Cys Lys Lys Pro Ala Glu Tyr Gly Tyr Val Ser Leu Gly Arg
    130                 135                 140

Leu Gly Ala Ala Lys Gly Ala Leu His Pro Gly Cys Phe Ala Pro Leu
145                 150                 155                 160

His Pro Pro Pro Pro Pro Pro Pro Pro Pro Ala Glu Leu Lys Ala Glu
                165                 170                 175

Pro Gly Phe Glu Pro Ala Asp Cys Lys Arg Lys Glu Glu Ala Gly Ala
            180                 185                 190

Pro Gly Gly Gly Ala Gly Met Ala Ala Gly Phe Pro Tyr Ala Leu Arg
        195                 200                 205

Ala Tyr Leu Gly Tyr Gln Ala Val Pro Ser Gly Ser Ser Gly Ser Leu
    210                 215                 220

Ser Thr Ser Ser Ser Ser Ser Pro Pro Gly Thr Pro Ser Pro Ala Asp
225                 230                 235                 240

Ala Lys Ala Pro Pro Thr Ala Cys Tyr Ala Gly Ala Gly Pro Ala Pro
                245                 250                 255

Ser Gln Val Lys Ser Lys Ala Lys Lys Thr Val Asp Lys His Ser Asp
            260                 265                 270
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Glu Tyr Lys Ile Arg Arg Glu Arg Asn Asn Ile Ala Val Arg Lys Ser
275 280 285

Arg Asp Lys Ala Lys Met Arg Asn Leu Glu Thr Gln His Lys Val Leu
290 295 300

Glu Leu Thr Ala Glu Asn Glu Arg Leu Gln Lys Lys Val Glu Gln Leu
305 310 315 320

Ser Arg Glu Leu Ser Thr Leu Arg Asn Leu Phe Lys Gln Leu Pro Glu
325 330 335

Pro Leu Leu Ala Ser Ser Gly His Cys
340 345

<210> 11
<211> 3038
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (724)..(2574)

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120

gcggacgcgt cccgatcttc ccctgtcccc accctgcccc gaccctcctc tccacctctc
180

gcgtcgtgac accagctggt aaatactccg ctgttcgtcc ctcaaaccct cggcagccag
240

ccgtgggcgt gagggagggt tctctctcct ctcgatgggg gtgttgcaaa cacagcgggg
300

agccccctgg taagggtccc cggtaaacgg gggagtcgca gctttttctc ttgctgctga
360

agtcgcccac gcaccatccg gggagtccta cggggaggga gcagagattt ttttttcccc
420

catattgctg ctgcttagta cgtgggcgat ggcagtgaga tggctcaggg aaggccgagg
480

aggccctggg taagcgaggg cttcgggggt tattttccca tttacacggc tccagagatc
540

ggcacaacat cttcctcctt tgctcctaaa cgttcctctt ctgggtaagg tttgggggat
600

cagggaagcc ccgggtttcc tgctgaaagg tgggggaagg gaacgtagac ctagagaggg
660

gggaattctt acagaaatcc tctttttttg gtcccttcta tttttcagtc tccggcagcc

720

tcc atg aaa gcc ctc aga ttg tcg gct tcc gcc ctc ttc tgc ctt ctg
768
Met Lys Ala Leu Arg Leu Ser Ala Ser Ala Leu Phe Cys Leu Leu
1 5 10 15

ctg atc aac ggg tta ggg gca gca ccc cct ggt cgc cct gag gcg cag
816
Leu Ile Asn Gly Leu Gly Ala Ala Pro Pro Gly Arg Pro Glu Ala Gln
20 25 30

cct cct cct ctc agc tct gag cat aaa gag ccg gta gcc ggg gac gca
864
Pro Pro Pro Leu Ser Ser Glu His Lys Glu Pro Val Ala Gly Asp Ala
35 40 45

gtg ccc ggg cca aag gat ggc agc gcc cca gag gtc cga ggc gct cgg
912
Val Pro Gly Pro Lys Asp Gly Ser Ala Pro Glu Val Arg Gly Ala Arg
50 55 60

aat tcc gag ccg cag gac gag gga gag ctt ttc cag ggc gtg gat ccc
960
Asn Ser Glu Pro Gln Asp Glu Gly Glu Leu Phe Gln Gly Val Asp Pro
65 70 75

cgg gcg ctg gcc gcg gtg ctg ctg cag gca ctc gac cgt ccc gcc tca
1008
Arg Ala Leu Ala Ala Val Leu Leu Gln Ala Leu Asp Arg Pro Ala Ser
80 85 90 95

ccc ccg gca cca agc ggc tcc cag cag ggg ccg gag gaa gaa gca gct
1056
Pro Pro Ala Pro Ser Gly Ser Gln Gln Gly Pro Glu Glu Glu Ala Ala
100 105 110

gaa gct ctg ctg acc gag acc gtg cgc agc cag acc cac agc ctc ccg
1104
Glu Ala Leu Leu Thr Glu Thr Val Arg Ser Gln Thr His Ser Leu Pro
115 120 125

gcg gcc gga gag ccc gag ccc gcg gcg ccc cct cgc cct cag act ccg
1152
Ala Ala Gly Glu Pro Glu Pro Ala Ala Pro Pro Arg Pro Gln Thr Pro
130 135 140

gag aat ggg ccc gag gcg agc gat ccc tcc gag gag ctc gag gcg cta
1200
Glu Asn Gly Pro Glu Ala Ser Asp Pro Ser Glu Glu Leu Glu Ala Leu
145 150 155

gcg tcc ctg ctc cag gaa ctg cga gat ttc agt cca agt agc gcc aag
1248
Ala Ser Leu Leu Gln Glu Leu Arg Asp Phe Ser Pro Ser Ser Ala Lys
160 165 170 175

cgc cag cag gag acg gcg gca gca gag acg gaa acc cgc acg cac acg
1296
Arg Gln Gln Glu Thr Ala Ala Ala Glu Thr Glu Thr Arg Thr His Thr
180 185 190

ctg acc cga gtg aat ctg gag agc ccg ggg cca gag cgc gta tgg cgc
1344
Leu Thr Arg Val Asn Leu Glu Ser Pro Gly Pro Glu Arg Val Trp Arg
195 200 205

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gct tcc tgg gga gag ttc cag gcg cgt gtc ccg gag cgc gcg ccc ctg
1392
Ala Ser Trp Gly Glu Phe Gln Ala Arg Val Pro Glu Arg Ala Pro Leu
        210                 215                 220

ccg ccc ccg gcc ccc tct caa ttc cag gcg cgt atg ccc gac agc ggg
1440
Pro Pro Pro Ala Pro Ser Gln Phe Gln Ala Arg Met Pro Asp Ser Gly
    225                 230                 235

ccc ctt ccc gaa acc cac aag ttc ggg gaa gga gtg tcc tcc ccc aaa
1488
Pro Leu Pro Glu Thr His Lys Phe Gly Glu Gly Val Ser Ser Pro Lys
240                 245                 250                 255

aca cac cta ggc gag gca ttg gca ccc ctg tcc aag gcg tac caa ggc
1536
Thr His Leu Gly Glu Ala Leu Ala Pro Leu Ser Lys Ala Tyr Gln Gly
                260                 265                 270

gtg gcc gcc ccg ttc ccc aag gcg cgc cgg gcc gag agc gca ctc ctg
1584
Val Ala Ala Pro Phe Pro Lys Ala Arg Arg Ala Glu Ser Ala Leu Leu
            275                 280                 285

ggc ggc tcc gag gcg ggc gag cgc ctt ctc cag caa ggg ctg gcg cag
1632
Gly Gly Ser Glu Ala Gly Glu Arg Leu Leu Gln Gln Gly Leu Ala Gln
        290                 295                 300

gtg gag gcc ggg cgg cgg cag gcg gag gcc acg cgg cag gcc gcg gcg
1680
Val Glu Ala Gly Arg Arg Gln Ala Glu Ala Thr Arg Gln Ala Ala Ala
    305                 310                 315

cag gaa gag cgg ctg gcc gac ctc gcc tcg gac ctg ctg ctc cag tat
1728
Gln Glu Glu Arg Leu Ala Asp Leu Ala Ser Asp Leu Leu Leu Gln Tyr
320                 325                 330                 335

ttg ctg cag ggc ggg gcc cgg cag cgc ggc ctc ggg ggt cgg ggg ctg
1776
Leu Leu Gln Gly Gly Ala Arg Gln Arg Gly Leu Gly Gly Arg Gly Leu
                340                 345                 350

cag gag gcg gcg gag gag cga gag agt gca agg gag gag gag gag gcg
1824
Gln Glu Ala Ala Glu Glu Arg Glu Ser Ala Arg Glu Glu Glu Glu Ala
            355                 360                 365

gag cag gag aga cgc ggc ggg gag gag agg gtg ggg gaa gag gat gag
1872
Glu Gln Glu Arg Arg Gly Gly Glu Glu Arg Val Gly Glu Glu Asp Glu
        370                 375                 380

gag gcg gcc gag gcg gcg gag gca gag gcg gac gag gcg gag agg gcg
1920
Glu Ala Ala Glu Ala Ala Glu Ala Glu Ala Asp Glu Ala Glu Arg Ala
    385                 390                 395

cgg cag aac gcg ctc ctg ttc gcg gag gag gag gac ggg gaa gcc ggc
1968
Arg Gln Asn Ala Leu Leu Phe Ala Glu Glu Glu Asp Gly Glu Ala Gly
400                 405                 410                 415
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gcc gag gac aag cgc tcc cag gag gag acg ccg ggc cac cgg cgg aag
2016
Ala Glu Asp Lys Arg Ser Gln Glu Glu Thr Pro Gly His Arg Arg Lys
                420                 425                 430

gag gcc gag ggg aca gag gag ggc ggg gag gag gag gac gac gag gag
2064
Glu Ala Glu Gly Thr Glu Glu Gly Gly Glu Glu Glu Asp Asp Glu Glu
            435                 440                 445

atg gat ccg cag acg atc gac agc ctc att gag ctg tcc acc aaa ctc
2112
Met Asp Pro Gln Thr Ile Asp Ser Leu Ile Glu Leu Ser Thr Lys Leu
        450                 455                 460

cac ctg cca gcg gac gac gtg gtc agc atc atc gag gag gtg gag gag
2160
His Leu Pro Ala Asp Asp Val Val Ser Ile Ile Glu Glu Val Glu Glu
    465                 470                 475

aag cgg aac cga aag aag aaa gcc cct ccc gag ccc gtg ccg ccc ccc
2208
Lys Arg Asn Arg Lys Lys Lys Ala Pro Pro Glu Pro Val Pro Pro Pro
480                 485                 490                 495

cgt gcc gcc ccc gcc ccc acc cac gtc cgc tcc ccg cag ccc ccg ccc
2256
Arg Ala Ala Pro Ala Pro Thr His Val Arg Ser Pro Gln Pro Pro Pro
                500                 505                 510

ccg ccc ccg tcc gca cga gac gag ctg ccg gac tgg aac gag gtg ctc
2304
Pro Pro Pro Ser Ala Arg Asp Glu Leu Pro Asp Trp Asn Glu Val Leu
            515                 520                 525

ccg ccc tgg gat cgg gag gag gac gag gtg tac ccg cca ggg ccg tac
2352
Pro Pro Trp Asp Arg Glu Glu Asp Glu Val Tyr Pro Pro Gly Pro Tyr
        530                 535                 540

cac cct ttc ccc aac tac atc cgg ccg cgg aca ctg cag ccg ccc tcg
2400
His Pro Phe Pro Asn Tyr Ile Arg Pro Arg Thr Leu Gln Pro Pro Ser
    545                 550                 555

gcc ttg cgc cgc cgc cac tac cac cac gcc ttg ccg cct tcg cgc cac
2448
Ala Leu Arg Arg Arg His Tyr His His Ala Leu Pro Pro Ser Arg His
560                 565                 570                 575

tat ccc ggc cgg gag gcc cag gcc cgg cac gcg cag cag gag gag gcg
2496
Tyr Pro Gly Arg Glu Ala Gln Ala Arg His Ala Gln Gln Glu Glu Ala
                580                 585                 590

gag gcg gag gag cgc cgg ctg cag gag cag gag gag ctg gag aat tac
2544
Glu Ala Glu Glu Arg Arg Leu Gln Glu Gln Glu Glu Leu Glu Asn Tyr
            595                 600                 605

atc gag cac gtg ctg ctc cgg cgc ccg tga ctgcccttcc cggtcccgcc
2594
Ile Glu His Val Leu Leu Arg Arg Pro
        610                 615

cccgcgcgcc cccgccgcgc gcgcgcgccg gcgccccccc ccgtgttgct ccccctcggt
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2654

gtttgcatgc gccccgccct gccccttgcc cctgtccccg ggctgcgtcg ggacctgcca
2714

gacccccctc ccgggtcctg agcccgaact cccagagctc acccgcgggt gaccggggcc
2774

agcccaggag ggcgggtggt ttgtgcgagt tcccttgcca cgcgccccgg ccccatcaag
2834

tcctctgggg acgtccccgt cggaaaccgg aaaaagcagt tccagttaat tgtgtgaagt
2894

gtgtctgtct gtcctcccag tcgggcctcc cacgagcccc tccagcctct ccaagtcgct
2954

gtgaatgacc ccttctttcc tttctctgtt gtaaataccc tcacggagga aatagttttg
3014

ctaagaaata aaagtgacta tttt
3038

<210> 12
<211> 616
<212> PRT
<213> Homo sapiens

<400> 12

Met Lys Ala Leu Arg Leu Ser Ala Ser Ala Leu Phe Cys Leu Leu Leu
1 5 10 15

Ile Asn Gly Leu Gly Ala Ala Pro Pro Gly Arg Pro Glu Ala Gln Pro
20 25 30

Pro Pro Leu Ser Ser Glu His Lys Glu Pro Val Ala Gly Asp Ala Val
35 40 45

Pro Gly Pro Lys Asp Gly Ser Ala Pro Glu Val Arg Gly Ala Arg Asn
50 55 60

Ser Glu Pro Gln Asp Glu Gly Glu Leu Phe Gln Gly Val Asp Pro Arg
65 70 75 80

Ala Leu Ala Ala Val Leu Leu Gln Ala Leu Asp Arg Pro Ala Ser Pro
85 90 95

Pro Ala Pro Ser Gly Ser Gln Gln Gly Pro Glu Glu Glu Ala Ala Glu
100 105 110

Ala Leu Leu Thr Glu Thr Val Arg Ser Gln Thr His Ser Leu Pro Ala
115 120 125

Ala Gly Glu Pro Glu Pro Ala Ala Pro Pro Arg Pro Gln Thr Pro Glu
130 135 140

Asn Gly Pro Glu Ala Ser Asp Pro Ser Glu Glu Leu Glu Ala Leu Ala
145 150 155 160

Ser Leu Leu Gln Glu Leu Arg Asp Phe Ser Pro Ser Ser Ala Lys Arg
165 170 175

Gln Gln Glu Thr Ala Ala Ala Glu Thr Glu Thr Arg Thr His Thr Leu
180 185 190

Thr Arg Val Asn Leu Glu Ser Pro Gly Pro Glu Arg Val Trp Arg Ala
195 200 205

Ser Trp Gly Glu Phe Gln Ala Arg Val Pro Glu Arg Ala Pro Leu Pro
210 215 220

Pro Pro Ala Pro Ser Gln Phe Gln Ala Arg Met Pro Asp Ser Gly Pro
225 230 235 240

Leu Pro Glu Thr His Lys Phe Gly Glu Gly Val Ser Ser Pro Lys Thr
245 250 255

His Leu Gly Glu Ala Leu Ala Pro Leu Ser Lys Ala Tyr Gln Gly Val
260 265 270

Ala Ala Pro Phe Pro Lys Ala Arg Arg Ala Glu Ser Ala Leu Leu Gly
275 280 285

Gly Ser Glu Ala Gly Glu Arg Leu Leu Gln Gln Gly Leu Ala Gln Val
290 295 300

Glu Ala Gly Arg Arg Gln Ala Glu Ala Thr Arg Gln Ala Ala Ala Gln
305 310 315 320

Glu Glu Arg Leu Ala Asp Leu Ala Ser Asp Leu Leu Leu Gln Tyr Leu
325 330 335

Leu Gln Gly Gly Ala Arg Gln Arg Gly Leu Gly Gly Arg Gly Leu Gln
340 345 350

Glu Ala Ala Glu Glu Arg Glu Ser Ala Arg Glu Glu Glu Glu Ala Glu
355 360 365

Gln Glu Arg Arg Gly Gly Glu Glu Arg Val Gly Glu Glu Asp Glu Glu
370 375 380

Ala Ala Glu Ala Ala Glu Ala Glu Ala Asp Glu Ala Glu Arg Ala Arg
385 390 395 400

Gln Asn Ala Leu Leu Phe Ala Glu Glu Glu Asp Gly Glu Ala Gly Ala

405 410 415

Glu Asp Lys Arg Ser Gln Glu Glu Thr Pro Gly His Arg Arg Lys Glu
420 425 430

Ala Glu Gly Thr Glu Glu Gly Gly Glu Glu Glu Asp Asp Glu Glu Met
435 440 445

Asp Pro Gln Thr Ile Asp Ser Leu Ile Glu Leu Ser Thr Lys Leu His
450 455 460

Leu Pro Ala Asp Asp Val Val Ser Ile Ile Glu Glu Val Glu Glu Lys
465 470 475 480

Arg Asn Arg Lys Lys Lys Ala Pro Pro Glu Pro Val Pro Pro Pro Arg
485 490 495

Ala Ala Pro Ala Pro Thr His Val Arg Ser Pro Gln Pro Pro Pro Pro
500 505 510

Pro Pro Ser Ala Arg Asp Glu Leu Pro Asp Trp Asn Glu Val Leu Pro
515 520 525

Pro Trp Asp Arg Glu Glu Asp Glu Val Tyr Pro Pro Gly Pro Tyr His
530 535 540

Pro Phe Pro Asn Tyr Ile Arg Pro Arg Thr Leu Gln Pro Pro Ser Ala
545 550 555 560

Leu Arg Arg Arg His Tyr His His Ala Leu Pro Pro Ser Arg His Tyr
565 570 575

Pro Gly Arg Glu Ala Gln Ala Arg His Ala Gln Gln Glu Glu Ala Glu
580 585 590

Ala Glu Glu Arg Arg Leu Gln Glu Gln Glu Glu Leu Glu Asn Tyr Ile
595 600 605

Glu His Val Leu Leu Arg Arg Pro
610 615